Cellular and Biochemical Events in Toll-like Receptor Signaling

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Abstract

In multicellular organisms, communication between cells relies on transmitting information across membrane barriers. Different cell types interrogate particular aspects of their surrounding environment through protein receptors that span membranes and upon ligand binding, trigger enzymatic signaling cascades that culminate in the activation of one or more transcription factors. Information transmission is bidirectional, as individual cells must be able to sense unique aspects of their surroundings, relay their specialized knowledge with others, and receive the collective knowledge of surrounding cells and tissues. This two-way communication is particularly important in the innate immune system, where potentially infectious organisms must be readily detected and identified, and their presence communicated to other cells in the vicinity. Because of the rapid generation time of microorganisms, delays between any of these steps - detection, information processing or information transmission - can make the difference between successful control of infection and pathogen outgrowth. For this reason, the receptors that identify potential pathogens must be able to detect pathogens wherever they are found, be exquisitely sensitive, and initiate a robust response. At the same time, the inflammatory response to infection is itself damaging. This requires that the same receptors are tightly controlled, both by modulating their sensitivity and by rapidly turning off responses through negative feedback pathways. Here, I show that the

toll/interleukin-1 receptor domain-containing adaptor protein (TIRAP) plays a critical role in controlling the sensitivity of toll-like receptor (TLR) signaling. First, TIRAP controls the assembly of the myddosome, a protein complex that activates signal transduction, from both the plasma membrane and within endosomes of macrophages. Though TIRAP's role at the cell surface was previously described, its endosomal function was previously unknown. Second, TIRAP is an important target for negative regulation. After stimulation with the TLR4 ligand lipopolysaccharide (LPS), macrophages induce a state known as endotoxin tolerance, in which they are refractory for additional LPS stimulation. Many mechanisms for endotoxin tolerance have been proposed, but here I show that TIRAP is degraded in endotoxin tolerance, and that the mechanism of TIRAP degradation also has implications for viral/bacterial superinfection.

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For Rachel,

who was there through everything.

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Chapter 1: Innate Immunity and Toll-like Receptor Biology

1.1 Introduction

Adaptive immunity

In the 200 years since Edward Jenner demonstrated that prior infection with cowpox could render an individual immune from smallpox, immunologists have expended a great deal of energy investigating how the immune system recognizes and responds to the presence of infectious microorganisms, and develops the immunological memory that makes such vaccination possible. Though Paul Ehrlich and Ilya Metchnikov shared the Nobel Prize in 1908 for their discoveries of antibodies and phagocytosis respectively, throughout most of the 20th century, there was a bifurcation in investigation of these types of immunity [1]. The immune processes typified by antibody production, carried out primarily by B- and T-lymphocytes, and involving *specific recognition* of pathogens by distinct groups of clonal receptors are collectively referred to as "adaptive immunity." The other branch, typified by phagocytosis and carried out primarily by myeloid cells, is referred to as "innate immunity," and involves *non-specific recognition* and elimination of microbes generally.

Adaptive immunity is made possible by receptors that are generated from random rearrangements of chromosomal DNA at specific genetic loci [2,3]. B-cell receptors (BCRs), which are produced in soluble form as antibodies upon B-cell activation, are capable of recognizing a functionally limitless number of chemical shapes [4]. In fact, B-cells may bind to synthetic molecules not produced in nature [5,6]. T-cell receptors (TCRs) are in principal equally diverse. However, during normal development, T-cells are

subjected to selective pressure such that only TCRs capable of binding to class 1 or class 2 major histocompatibility complexes (MHC-I and MHC-II) are provided with survival signals [7]. Mature T-cells therefore recognize antigenic peptides that are presented in grooves of MHC molecules and thus are considered "MHC restricted."

During a productive immune response, T-cells and B-cells capable of binding to ligands of the offending microbe are activated and undergo clonal expansion - a rapid series of mitotic divisions on a per-cell basis [8,9]. Because their receptors are rearranged at the level of the chromosome, this clonal expansion produces a large population of identical cells, all capable of recognizing the current infectious organism. In the case of B-cells, clonal expansion is accompanied by affinity maturation, in which activation-induced cytidine deaminase (AID) introduces mutations to the locus of the binding site of the BCR [10]. Those mutations that increase affinity of the resultant gene product are positively selected, while those clones that express mutations that decrease affinity are deleted from the repertoire. Though the antibodies present at the end of this process are different and in principal better at binding to the infectious agent, these antibodies can still be considered "clonal" since they arose from the same set of chromosomal rearrangements.

Once an infection has been resolved, previously clonally expanded T- and B-cells undergo a contraction phase, but a minority differentiate to display a "memory" phenotype that are capable of rapidly responding to subsequent infectious challenge of the same microbe [11]. These memory cells are also responsible for the success of vaccination, since exposure to dead or weakened versions of an infectious agent can

still generate a productive memory response that will respond upon encounter with the actual infectious agent. Though many details of these processes had yet to be experimentally resolved, much of the underlying framework for understanding adaptive immunity and the generation of clonal populations of T- and B-cells with unique receptors had been elucidated by the penultimate decade of the 20th century [1].

The Janeway Hypothesis

It was at this stage of development in immunological understanding that Charles Janeway addressed the Cold Spring Harbor Symposium on the immune system in 1989, stating

> *I believe it is safe to state that our understanding of immunological recognition is approaching some sort of asymptote, where future experiments are obvious, technically difficult to perform, and aim to achieve ever higher degrees of precision rather than revolutionary changes in understanding.* [12]

This claim has largely been vindicated by the following twenty five years of immunology research into T-cell and B-cell recognition of their cognate ligands. However, Janeway also introduced an apparent conundrum. Previously, immunologists believed that adaptive immune self/nonself discrimination was conferred during T- and B-cell development, when lymphocytes with self-reactive receptors would be deleted from the repertoire [1,12]. According to this clonal selection theory, this process of negative selection (now called central tolerance) was sufficient to establish the immunological distinction between self and nonself. Thus, all foreign molecules should be equal with

respect to immunogenicity. However, Janeway noted that a foreign protein or peptide alone could not initiate an adaptive immune response, and that immunization requires inclusion of adjuvants - molecules from dead microorganisms - to elicit activation of T- and B-cells, antibodies, antigen-specific killing, and memory [12].

In addition to the activating signal provided by BCR or TCR binding to their cognate ligand (signal 1), immunologists knew that B-cells and T-cells required a signal from antigen presenting cells (in the case of T-cells) or from previously activated helper T-cells (in the case of B-cells) [9]. This so-called "signal 2" was not provided by naïve cells, but several microbial molecules could imbue myeloid cells like dendritic cells with the ability to provide this co-stimulation. Janeway hypothesized that potentially infectious microorganisms might present the immune system with chemical patterns that would identify when an infection was underway. The presence of these pathogen-associated molecular patterns, or PAMPs, would elicit signal 2, while the absence of PAMPs would be a cue that any activated B- or T-cells receiving signal 1 alone were likely auto-reactive.

In order to be proper determinants of infection, Janeway hypothesized that PAMPs would have to be common to large numbers of infectious microbes, since by definition, there would be a limited number of receptors that could respond to them [12]. Furthermore, these ligands must be unique to microorganisms, since ligands present in the absence of potential infection could generate inflammation and adaptive immune responses to self or to innocuous proteins. Janeway also proposed a theoretical framework for the receptors that would recognize PAMPs, which he called pattern recognition receptors or

PRRs. In contrast to B- and T-cell receptors, which are expressed on specialized cells and are randomly generated, PRRs should, he argued, be encoded in the germline and evolutionarily selected to recognize only microbial ligands. In addition, these receptors should be expressed on multiple cell types to detect pathogens in any context.

Thus, Janeway provided a conceptual link between innate and adaptive immunity, and the experimental evidence of the last quarter century have confirmed this hypothesis. Mammalian immune cells express many different classes of PRRs, which recognize a diverse set of PAMPs (see Chapter 1.3), and it is clear that PRRs are critical in the activation of adaptive immune responses through induction of inflammation.

Inflammation

The clinical signs of inflammation were famously described by the Roman physician Celsus as *Dolor*, *Calor*, *Rubor* and *Tumor*, or pain, heat, redness and swelling [13]. Today, we know that these symptoms are associated with activities of the immune system that promote pathogen control and clearance. The modern molecular definition of inflammation refers to the production of particular chemical messengers such as cytokines and chemokines, which are secreted in response to tissue damage or infection [14,15]. These proinflammatory molecules promote pathogen clearance in a number of ways; they upregulate phagocytosis, increase vascular permeability, recruit monocytes and lymphocytes from circulation, up-regulate antigen presentation and co-stimulatory molecules for activating the adaptive immune system, and have direct effects on skewing adaptive immune cell differentiation.

Some cytokines have pleiotropic effects, both on local tissues and systemically. For example, in response to recognition of PAMPs, macrophages produce and secrete the cytokine tumor necrosis factor α (TNFα) [16]. Locally, TNFα can induce infected cells (or cells under other types of stress) to undergo programmed cell death [17]. Vascular endothelial cells respond to TNFα by loosening tight junctions to increase vascular permeability[18,19], permitting increased immune cell recruitment, as well as increasing expression of adhesion molecules [20]. TNFα can also activate dendritic cells [21] and macrophages [22], causing numerous transcriptional changes including production of other proinflammatory cytokines by macrophages. TNFα signaling also increases expression of prostaglandin E2 (PGE2) [23], which circulates to the hypothalamus and induces fever.

Other cytokines induced by PRRs have more direct effects on the outcomes of activation of the adaptive immune system. For example, macrophages and other myeloid cells also produce IL-12 in response to PAMP stimulation [24,25]. Though IL-12 can have local effects, such as activation of natural killer (NK) cells [26], the primary role of IL-12 is thought to be in skewing the differentiation of naïve CD4+ T-cells towards a Th1 phenotype [27]. Thus, these cytokines may be considered "signal 3," complementing clone-specific recognition of ligand (signal 1) and PAMP-dependent co-stimulation (signal 2). Several cytokines combine local innate functions and effects directly on T- and B-lymphocytes. Like TNFα, IL-6 is a pyrogen [28], and acts on vascular endothelium to induce permeability [29]. However, IL-6 also plays roles in CD4 T-cell differentiation, acting in concert with transforming growth factor β (TGFβ) to permit T helper 17 (Th17) skewing [30].

Chemokines are a subset of immune signaling molecules that are responsible for mediating chemotaxis, or the movement of cells [14]. Like cytokines, chemokines can be generally divided into those that primarily act locally to promote pathogen clearance, and those that primarily mediate T-cell and B-cell function. Also as with cytokines, there is significant overlap in these categories. IL-8 (also called neutrophil chemotactic factor) is a potent chemoattractant for circulating neutrophils and promotes inflammation [31], while CCL19/CCL21 and CXCL13 establish gradients in order to divide lymph nodes into T- and B-zones respectively [32]. Naïve CD4 T-cells express the chemokine receptor CCR7, which binds CCL19/21 and allows the cells to home to the T-zone [33]. Differential expression of chemokine receptors on immune cells can have dramatic functional consequences, as when activated CD4 T-cells stop expressing CCR7, and upregulate CXCR5, allowing them to move into the B-cell zone where they provide help to activated B-cells [32].

Interestingly, production of cytokines and chemokines (or chemokine receptors) that act locally to promote inflammation and those that act distally to permit activation of adaptive immunity are often differentially regulated. Inflammation may occur in the absence of microbes, but for Janeway's hypothesis to be correct, that inflammation should not lead to a productive adaptive immune response. Indeed, while TNFα may be produced in response to non-microbial stimuli such as sterile tissue damage [34], IL-12 is typically only produced downstream of the activation of PRRs. Of note, TNFα alone may activate dendritic cells to produce signal 2 co-stimulatory molecules, but in the absence of signal 3, no productive differentiation can occur. Thus, inflammation has at least two molecular states - that induced by PAMPs, and provides signal 2 and signal 3, and that

associated with non-microbe induced inflammation that does not provide signal 3. This latter non-infectious inflammation may indicate danger, but not infection, and the instigating signals are called "danger-associated molecular patterns" or DAMPs.

1.2 Macrophages and Other Professional Microbe Detectors

Many cell types can sense potentially infectious microorganisms and signal the presence of those microbes to surrounding cells. However, the majority of these cell types are only capable of sensing an infection within themselves, through receptors that interrogate the cytosol, and secrete a limited array of cytokines and chemokines. A much smaller number of cell types express the larger suite of receptors that enable detection of pathogens wherever they are found, and are capable of initiating a robust inflammatory response. In particular, the professional phagocytes - macrophages, dendritic cells and neutrophils - express many classes of PRRs, and are capable of activating the adaptive immune system both through cytokines and through the expression of costimulatory molecules.

Macrophages

Cells that could engulf and destroy microbes were first described by Ilya Metchnikov in the early twentieth century in starfish embryos. We now know that these phagocytes (literally: eating cells) play important roles in both pathogen clearance and wound repair. This is somewhat paradoxical, in that pathogen clearance necessitates a pro-inflammatory response, but inflammation itself can damage tissues. Therefore, resolving a wound requires an anti-inflammatory state [35]. The dual roles of macrophages in pro- and anti-inflammatory responses may be best understood as promoting tissue

homeostasis. In order to recover from perturbations caused by injury, the first priority must be to clear potentially infectious microorganisms, since pathogens may represent a persistent threat to tissue health if allowed to persist. In this circumstance, an inflammatory response is appropriate, though additional damage from inflammation may occur. Indeed, the first stage of wound healing is characterized by inflammation and infiltration of leukocytes [35]. Once the threat of infection is cleared however, continued inflammation is deleterious, and an active anti-inflammatory response must ensue to heal the initial injury and prevent further damage.

Macrophages are tissue-resident phagocytes that are present at steady state in almost every tissue. Though given different names depending on location, macrophages in all tissues share features that make them distinct from other cell types. Lineage tracing studies suggest that bone marrow stem cells differentiate into monocytes, which leave the bone marrow and circulate in blood [36]. Monocytes are recruited into tissues from the blood and may differentiate further into myeloid dendritic cells or macrophages.

As implied by their name (literally: big eater), macrophages are highly phagocytic. They express a broad range of scavenger receptors that allow them to bind to apoptotic cells, cell debris, infectious microorganisms and complement-opsonized particles [37]. Their phagocytic ability even extends to inorganic materials such as uric acid crystals [38], carbon nanotubes [39], and tattoo ink [40]. Additionally, the phagocytic compartment of macrophages is superbly degradative, and they may be identified by high expression of several lysosomal enzymes, such as lysozyme M [41]. Though other cell types (such as neutrophils) that share these phagocytic and degradative abilities may be recruited to

tissues under inflammatory conditions, macrophages are unique in being present in tissues at steady state.

Though the ability of macrophages to engulf and destroy unwanted materials plays a role in the normal functioning of tissues, immunologists primarily consider this feature in its pathogen-clearing context. Yet the immunological role of macrophages does not end at direct microbial clearance (Figure 1.1). These cells also possess a wide variety of other effector functions important to the immune system. Depending on tissue type, macrophages may express every known pattern recognition receptor thus far described (See Chapter 1.3), and produce large amounts of cytokines and chemokines in response to PAMPs. Macrophages also express both Class-I and Class-II MHC and when activated, the T-cell costimulatory molecules CD80 and CD86 [42], allowing them to function as antigen presenting cells for the maintenance of adaptive T-cell responses in an infected tissue.

This diversity of macrophage function extends even further, as their behavior may be modified by the cytokine milieu in their vicinity. Under steady-state conditions, macrophages are thought to exhibit a naïve or M0 phenotype [43], characterized by active phagocytosis and macropinocytosis, and non-inflammatory clearing of apoptotic cells. In response to the cytokine interferon-γ (IFNγ), these cells differentiate into a “classically activated” or M1 state [44-46]. M1 macrophages increase phagocytosis, increase lysosomal degradation by up-regulating nucleases and proteases, become more bactericidal due to increased production of reactive oxygen species (ROS) through

Figure 1.1

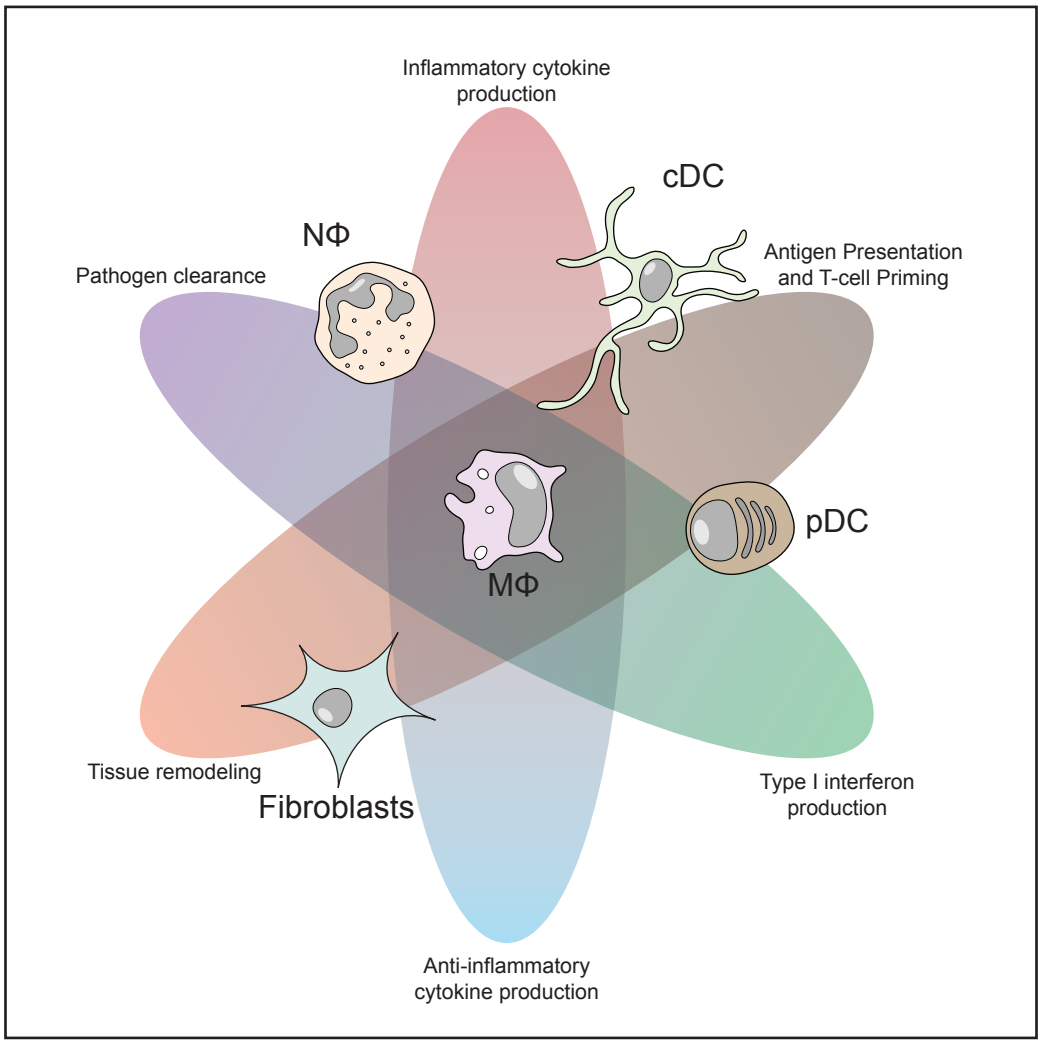

Figure 1.1 Macrophages are responsible for tissue homeostasis.
Macrophages (MΦ) participate in microbial clearance, inflammatory cytokine and interferon production, and other immune-activating activities. However, they may also produce anti-inflammatory cytokines, and engage in tissue remodeling to promote wound healing. Other immune cells like neutrophils (NΦ), classical dendritic cells (cDC) and plasmacytoid dendritic cells (pDCs) are more specialized for a subset of these functions.

expression of inducible nitric oxide synthase (iNOS), and produce more cytokines in response to PAMPs.

"Alternatively activated" or M2 macrophages were initially described [47] as the opposite of M1 polarization, reflecting the distinction between type-1 and type-2 T-helper cell phenotypes. The inducer of M1 differentiation, IFNγ is a prototypical Th1 cytokine, while M2 differentiation can be accomplished with the Th2 cytokines interleukin 4 (IL-4) and IL-13 [43]. In addition, M2 macrophages express arginase, which antagonizes ROS production by diverting the substrate arginine away from iNOS, reminiscent of the mutually exclusive expression of opposing transcription factors in T-helper cell differentiation. However, it has become increasingly clear that this simple dichotomy is not sufficient to describe macrophage polarization. First, several different stimuli seem to induce "alternative" activation and the expression of the signature arginase, yet these stimuli do not result in identical macrophage phenotypes. Some have suggested sub-designations of M2 for IL-4/IL-13 induced (M2a), immune-complex induced (M2b), and IL-10 induced (regulatory macrophages) but these classifications do not reflect the full complexity of macrophage differentiation [46].

Second, the term "differentiation" may not even be appropriate, as the plasticity of these phenotypes *in vivo* has not been readily established. T-helper cell differentiation is largely uni-directional, as transcription factors called "master regulators" are necessary for expressing Th1, Th2, Th17 and Treg effector functions [48-52], and these master regulators largely preclude expression of the others [53] (though even with T-helper cells, evidence of some plasticity continues to mount [54,55]). No equivalent master regulators

have been described for macrophages. Further, while T-cells have clonally selected unique receptors and are likely to be required only in response to a single type of infection, as described above, macrophages have many diverse functions in the same tissue. While it is possible that activated macrophages might execute their effector function and then die, to be replaced by newly differentiated monocytes, experimental evidence suggests that tissue-resident macrophages are incredibly long-lived [56,57].

Further complicating these classifications, as discussed above, macrophages have important roles in homeostasis. Capable of expressing matrix metalloproteases and other extracellular matrix (ECM) modifying enzymes [58,59], macrophages home to sites of tissue injury where they eliminate dead or dying cells and assist in reorganizing the tissue substratum in order to facilitate healing. Though I primarily focus on macrophages' ability to promote inflammation and immune responses in this document, during wound healing, they also play an important role in suppressing immune responses to reduce inflammatory tissue damage. For example, in a mouse model of wound healing, specific depletion of macrophages caused prolonged neutrophil infiltration, increased inflammation, and decreased neoangiogenisis and wound contraction [60]. These macrophages have in some cases been classified as regulatory macrophages or M2, yet the extent of the overlap is unclear [43]. The anti-inflammatory roles of macrophages have also been extensively documented in the context of tumors, where so-called "tumor-associated macrophages" have a phenotype similar but distinct from wound healing and M2 macrophages and may suppress the T-cells to kill transformed cells [61,46].

Due to the diversity of functions of these cells, studying their role in innate immunity *in vivo* has a number of challenges. In this regard, *in vitro* differentiated primary bone marrow-derived macrophages (BMDM) are an incredibly useful tool to study PRRs. These cells are relatively easy to differentiate to high purity in tissue culture, initiate robust responses to PRR ligands, and can be efficiently transfected by nucleofection. In addition, these cells are uniformly naive, while macrophages and monocytes isolated from living animals may have previous exposure to PAMPs and activating cytokines. Despite the usefulness of these cells for experimental studies of toll-like receptors (TLRs), many biochemical approaches are problematic, as ectopic expression of many genes involved in TLR signaling induces spontaneous activation. For this reason, immortalized BMDM are a useful stand-in for studying the same processes [62,63]. One can create immortalized cells of the same genetic backgrounds as primary cells [64], and stably transduce these cells via retroviral gene insertion, allowing selection for low levels of expression through the use of selectable markers [65].

Neutrophils

Neutrophils are professional phagocytes that are recruited from circulation to inflamed tissues. This recruitment is mediated by chemokine and integrin signaling, initiated by tissue-resident cells [66]. Unlike macrophages, which have several homeostatic roles, neutrophils are specialized for pathogen clearance, with a high rate of phagocytosis, high degradative capacity, and expression of many PRRs that are capable of inducing inflammatory cytokine production [67,68]. Neutrophils also have the ability to undergo a unique form of programmed cell death that aids in the clearance of pathogens [69]. Upon

microbial encounter, neutrophils may expel their genomic material in the direction of microbes, trapping them in DNA. These neutrophil extracellular traps (NETs) are microbicidal, and are important for clearing several extracellular bacterial infections.

Classical Dendritic Cells

Classical dendritic cells (cDCs), like macrophages, are tissue-resident phagocytes and are found in most tissue contexts, but like neutrophils, the primary role of cDCs is immunological, rather than homeostatic [70]. However, though cCDs are capable of detecting microbial ligands and of producing inflammatory cytokines in response, unlike macrophages and neutrophils, cDCs do not play a significant role in direct pathogen clearance. Instead, cDCs seem to endocytose particles for the express purpose of presenting them to cells of the adaptive immune system. Upon activation by inflammatory cytokines like TNFα or to PAMPs, tissue-resident cDCs migrate to tissue-draining lymph nodes in order to present local antigens to T-cells [71].

Plasmacytoid Dendritic Cells

Though named dendritic cells due to expression of the cell surface marker CD11c [72], plasmacytoid dendritic cells pDCs are functionally and developmentally distinct. The name itself is a contradiction, as both "plasmacytoid" and "dendritic" refer to opposing morphological features, and the stem cell lineage that gives rise to pDCs diverges from that of other myeloid cells very early, as evidenced by early presence of 9-O acetylated sialic acid (Netrevali I, thesis defense. Harvard University, 2014). Though pDCs have been shown to be capable of antigen presentation and T-cell priming [73], efficient

purification of pDCs from cDCs has been challenging, and some of the activity may be explained by contaminating cDCs.

Plasmacytoid DCs are specialized to detect viruses and produce copious levels of type-I interferon (IFN) [74,75]. Of note, pDCs are unresponsive to cell-surface TLR ligands (see Chapter 1.5), but have a specialized response to endosomal TLRs. Like macrophages, detection of viral nucleic acid by TLR7 and TLR9 in pDCs leads to the activation of NFκB and pro-inflammatory cytokine production. However, pDCs uniquely activate an additional signaling cascade that culminates in the activation of IRF7 [76,77]. Though macrophages are capable of activating this pathway when ligands are artificially delivered to the proper subcellular compartment [78], the physiological relevance of this pathway in macrophages is uncertain (See Chapter 1.5).

1.3 Toll-like Receptors and other PRRs

In order for the cells of the innate immune system to clear pathogens or activate the adaptive immune system, the first step is detecting that a pathogen is present. As Charles Janeway predicted over twenty years ago [12], germline encoded PRRs detect PAMPs to induce inflammation and activate adaptive immunity. In the past twenty years, immunologists have identified and characterized many such PRRs. PRRs exist in diverse locations within the cell, recognize a diverse set of ligands and activate a diverse set of signaling pathways and transcription factors. However, these PRRs all fit within the conceptual framework delineated by Janeway in 1989.

Mammalian PRRs can be classified along several criteria, with the most obvious delineation separating transmembrane receptors that survey the extracellular milieu for

the presence of potentially infectious microorganisms, and the intracellular receptors that are poised to detect the presence of actual infections within an individual cell (Figure 1.2) [79]. The former group, consisting of toll-like receptors (TLRs) and c-type lectin receptors (CLRs), are largely restricted to professional immune myeloid cells such as macrophages and dendritic cells. Since any cell can in principal be infected, the later group, whose two main families are the RIG-I-like receptors (RLRs) and the NOD-like receptors (NLRs), are found expressed at steady state in most cell types, though their expression can be substantially enhanced when cells are exposed to several inflammatory cytokines.

Toll-like Receptors

The earliest discovered and still best characterized PRRs are the toll-like receptors (TLRs). The gene *toll* was initially described in the fruit fly *Drosophila melanogaster* as being involved in development during dorsal-ventral patterning in fly embryos [80]. In 1996, Bruno Lemaitre in Jules Hoffman's lab showed that Toll was involved in the immune recognition of fungal pathogens in flies [81], a discovery for which Hoffman was granted the Nobel Prize in 2011. Toll signaling leads to activation of Dorsal, the homologue of mammalian nuclear factor kappa B (NFκB). During development, there is a gradient of Toll signaling that establishes the dorsal-ventral axis of the embryo, but in the adult fly, the receptor detects fungal pathogens and induces expression and secretion of antimicrobial peptides such as drosomycin and metchnikoin.

Nicholas Gay and Fionna Keith recognized that the intracellular domain of *Drosophila* toll was similar to that of mammalian interleukin-1 receptor (IL-1R) [82]. Since activation of

Figure 1.2

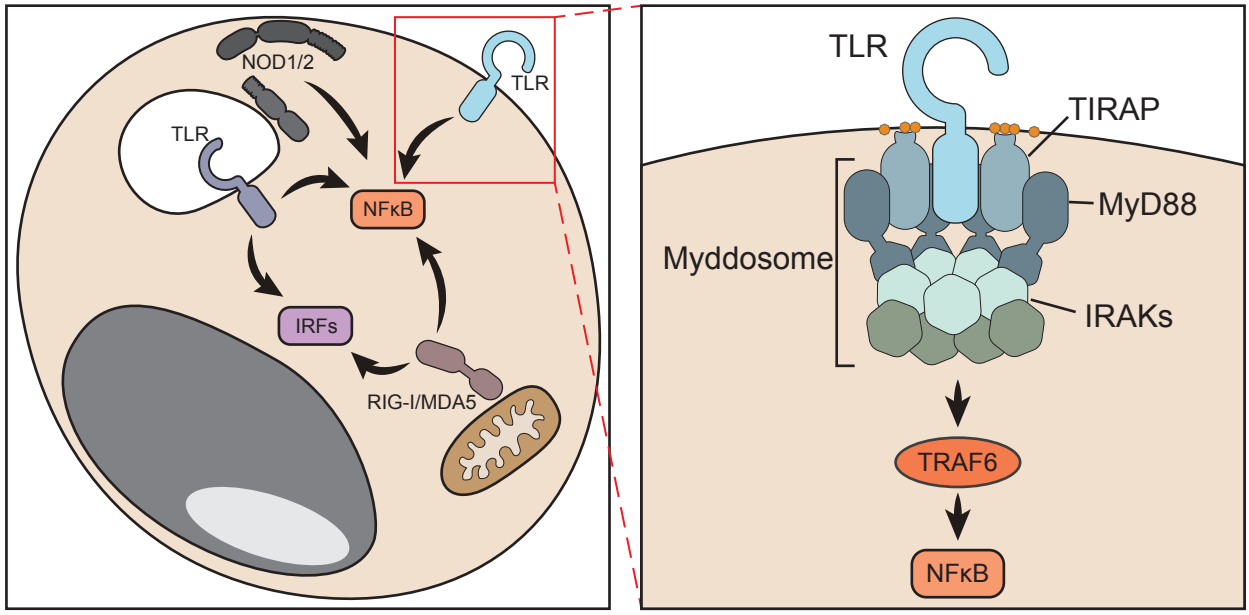

Figure 1.2 Pattern Recognition Receptor and Toll-like Receptor Signaling
PRRs trigger signaling cascades that lead to the activation of many transcription factors, including NFκB and IRFs (left). TLRs in particular (right) utilize the adaptor proteins TIRAP and MyD88 to recruit IRAKs in a large multimeric protein complex called the myddosome. IRAKs activated in the myddosome are capable of triggering a signaling cascade culminating in the activation of NFκB and AP-1.

IL-1 in mammals led to inflammation and co-stimulatory activation, Charles Janeway and Ruslan Medzhitov suspected that a mammalian homologue of Toll might be an example of the pattern recognition receptors Janeway hypothesized. They subsequently showed that a constitutively active mutant of "human toll" (now TLR4) was capable of activating NFκB and co-stimulatory molecules in macrophages [83]. Meanwhile, Alexander Poltorak in Bruce Beutler's lab was attempting to map a well-known mutation in mice that rendered the strain insensitive to bacterial lipopolysaccharide (LPS). They showed that this mutation mapped to the locus encoding the murine equivalent of human Toll [84]. This series of studies finally demonstrated the existence of a bona fide PRR and its cognate PAMP.

Mammalian TLRs are single-pass transmembrane receptors with a leucine-rich repeat (LRR) ectodomain and a cytosolic toll/IL-1 receptor (TIR) domain. Upon ligand binding and receptor dimerization, TLRs recruit a series of adaptor proteins through heterotypic TIR-TIR interactions (Figure 1.2), which in turn activate signaling cascades that culminate in the activation of several transcription factors, including NFκB, AP-1 and interferon regulatory factors (IRFs) (see also Chapter 1.4). Most TLRs form homodimers [85-88], though TLR2 instead forms heterodimers with TLR1 and TLR6 [89,90].

TLRs may be conceptually divided based on subcellular location of ligand binding. TLRs 1, 2, 4, 5 and 6 first encounter their ligands at the cell surface, while TLRs 3, 7-9, and 11-13 encounter their ligands in degradative endosomal compartments [91]. This bifurcation in localization mirrors differences in the ligands for these receptors. Cell-surface TLRs detect structural components of viruses, bacteria [92,93] and fungi, such as

LPS from the outer membrane of gram-negative bacteria (TLR4) [84], lipoproteins from gram positive bacteria (TLR2)[94], and bacterial flagellin (TLR5) [95]. By contrast, endosomal TLRs detect primarily nucleic acid ligands from microbial sources, such as unmethylated CpG-motif containing DNA (TLR9), single-stranded RNA (TLR7) [96] and ribosomal RNA (TLR13) [97,98]. Neither the localization nor the ligand of TLR10 have been described. However, TLR10 was recently shown to act in concert with TLR2 in response to *Listeria monocytogenes* [99], suggesting that it may heterodimerize with TLR2 at the cell surface, analogously to TLR1 and TLR6. For more on the cell biology of TLRs, see chapter 1.5.

RIG-I Like Receptors

The RLR family consists of three members, the name-sake retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated 5 (MDA-5), and RIG-I-like receptor 3 (RLR-3) [100]. All three of these RLRs contain RNA-helicase domains and bind RNA ligands in the cytosol. The precise nature of these ligands is unknown, though RIG-I seems to preferentially bind to uncapped 5' triphosphate RNAs of viral origin [101], while MDA-5 is thought to bind long branched RNAs, indicative of viral replication intermediates [102]. Though these ligands typically originate from viruses with RNA genomes, some DNA viral genomes may be transcribed into RNA by RNA polymerase III, producing RNA ligands that can be recognized by RIG-I [103,104].

Like TLRs, RLRs also rely on a receptor-proximal adaptor protein to initiate signal transduction, but in contrast to TLRs, which are transmembrane receptors and utilize non-membrane adaptor proteins, RLRs are cytosolic proteins that use a transmembrane

adaptor. Mitochondria-associated anti-viral signaling protein (MAVS), is associated with the membranes of mitochondria, peroxisomes, and mitochondrial associated membranes (MAMs) [62,100,105]. The cell biology of RLR signaling remain largely unclear, but upon ligand binding, RIG-I and MDA-5 are thought to associate with MAVS via homotypic interactions of their respective caspase activation and recruitment domains (CARDs). MAVS then forms prion-like aggregates [106] that seem to be required for further activating Tank-binding Kinase 1 (TBK1), leading to the phosphorylation and activation of IRF3 and the production of type-1 IFN. MAVS may also activate several members of the TRAF family of ubiquitin ligases, leading to the activation of NFκB and pro-inflammatory cytokine production [107]. RLR-3 does not have a CARD domain, and it is unclear whether it can participate in signaling alone, but seems to modulate the abilities of RIG-I and MDA-5 to trigger responses to certain ligands [108].

Because all cells may be infected with viruses, RLR's have ubiquitous expression in most cell types that have been studied [109]. Whenever a cell is infected, it is advantageous to initiate an antiviral response in the surrounding tissue, thus, most cells responding via RLRs are capable of producing type-I IFN. Detection of type-I IFN in surrounding cells induces expression of many anti-viral genes, including RLRs themselves, increasing the likelihood of timely detection should the infection spread. In addition, professional immune cells such as macrophages may produce proinflammatory cytokines, inducing a more robust immune response and the activation of the adaptive immune system [62,110].

NOD-Like Receptors

The nucleotide oligomerization domain containing proteins (NOD1 and NOD2) are the prototypical members of a large class of intracellular proteins called the NOD-like receptors (NLRs) [111]. NLRs are defined by the presence of a central NACHT domain, at least one leucine-rich repeat domain, and a variable C-terminal protein-protein interaction domain that may be a CARD (NLRCs) or a PYRIN domain (NLRPs). A small number of NLRs have a different C-terminal effector domain (NLRXs). The functions of NLRs are also quite diverse; in fact, not all NLRs are actually receptors. For example, CIITA is expressed in the nucleus of antigen presenting cells and is a transactivator of MHC class II expression [112]. NLRC5 may play a similar role in MHC-I expression [113], though several other functions have been ascribed to it as well [114-116]. The most PRR-like NLRs are NOD1/2, which recognize bacterial outer membrane components found in the cytosol, and activate a signal transduction pathway culminating in the production of pro-inflammatory cytokines. Though long thought to be strictly cytosolic, NOD1 and 2 are capable of recruiting autophagy factors to the plasma membrane [117]. Localization to a site of bacterial entry makes sense, though whether signal transduction occurs from this location is unclear [79,91].

Several other NLRs (NLRP1, NLRP3, and NLRC4) are also involved in signaling the presence of infectious microorganisms, but their status as true PRRs is unclear. Upon encountering their ligands, this group of NLRs act to assemble a large, multi-protein enzymatic structure known as the inflammasome, which culminates in the activation of caspase-1, the cleavage of pro-IL-1β and secretion of mature IL-1β. [118,119] For example,

upon activation, NLRP3 associates with the adaptor protein ASC, and forms a wheel-like inflammasome oligomer [120]. Both NLRP3 and ASC contain CARD domains, which are capable of recruiting pro-caspases [38,121]. Caspases are proteases that are first produced as proenzymes, which have very low enzymatic activity until proteolytically cleaved. The formation of the inflammasome positions the procaspases such that they can act upon each other, causing full activation [121].

The exact ligand(s) of NLRP3 are unclear, though several organic and inorganic stimuli, especially those that cause breaches in endosomal membrane integrity, are capable of activating it. The ligands of NLRP1 and NLRC4 are similarly obscure, and it is unclear whether these inflammasome-activating proteins truly recognize microbial ligands (PAMPs) directly, or if they merely recognize the signatures of microbe-induced damage. In particular, NLRC4 appears to act downstream of another NLR NAIP5, which detects cytosolic bacterial flagellin [122]. Thus, NLRC4 may be thought of as an adaptor protein rather than a true PRR.

An additional feature sets inflammasome activators apart from other PRRs: they do not appear to be capable of activating signal transduction pathways culminating in cytokine expression on their own. Inflammasome activation leads to the processing and secretion of IL-1β and IL-18, but transcription and translation of these cytokines are dependent on signals from other PRRs [63,119]. For this reason, and because endogenous expression of inflammasome-activating proteins is quite low in resting cells, experimental investigation of these pathways is typically performed after treatment with TLR ligands, making definitive statements about the inflammasome's role in these

pathways complicated. An exception to this rule is AIM2, which is not itself a member of the NLR family, but which has been shown to bind directly to DNA, and is capable of activating both gene transcription and inflammasome activation in the absence of other PRRs [123,124].

Other putative PRRs

Like the inflammasome-activating NLRs, the status of c-type lectin receptors (CLRs) as bona fide PRRs is contentious. CLRs are expressed primarily on DCs, and recognize carbohydrate ligands [125]. Though CLRs may bind to endogenous or microbe-associated ligands, only those that bind exclusively foreign carbohydrates could be considered PRRs. The best characterized consequences of CLR activation in the innate immune system do not involve direct activation of gene transcription. Instead, CLR activation seems to modify the response of other PRRs. For example, DC-SIGN (CD209) binds to mannose and fucose ligands found on several types of bacteria and fungi, and leads to phosphorylation and acetylation of the p65 subunit of NFκB, increasing its binding affinity for several target genes, and leading to (among other things) enhanced TLR-driven IL-10 production [126]. Though this activity itself is TLR-independent, actual transcription of IL-10 cannot proceed in the absence of other signaling pathways mediated by true PRRs. By contrast, several CLRs, including Dectin-1 have been shown to have the ability to induce transcription of TNFα and other cytokines directly, including those that influence T-cell activation and differentiation, suggesting that these receptors may be bona fide PRRs [127]. It is possible that the large family of CLRs may be

similar to NLRs, in that some members act as PRRs in the classical sense, while others perform other immunomodulatory roles.

Several other classes of proteins have been characterized that may claim to be members of the PRR pantheon, such as the cytosolic sensor of DNA called cyclic GMP-AMP synthase (cGAS) [128,129]. Upon binding to viral DNA, cGAS produces a cyclic dinucleotide second messenger, which is capable of initiating NFκB activation and type-1 IFN production. Other putative cytosolic DNA sensors (CDS) such as AIM2 (see previous section) and IFI16 may play roles in sensing cytosolic viral DNA, though their role may be less important or cell-type specific [123,124,130,131].

1.4 TLR Biochemistry

Despite the growing understanding of many of these PRRs and their roles in pathogen detection, TLRs remain the most well characterized. In contrast to most transmembrane receptors, which initiate enzymatic signaling cascades through direct activation of signaling enzymes such as kinases, innate immune receptors and some proinflammatory cytokine receptors (such as TNFα receptor and IL-1R) do not engage directly with signaling enzymes, and instead rely on adaptor proteins. These adaptors have no enzymatic function, but instead act as a bridge between the receptor and downstream enzymes. Toll-like receptors in particular utilize not one, but two adaptor proteins: the sorting adaptors, which are hard-wired to the subcellular compartment where signaling is initiated, and the signaling adaptors, which bridge receptor and sorting adaptor to signaling enzymes.

Signaling Adaptors

All TLRs are single-pass transmembrane receptors consisting of a leucine-rich repeat (LRR) ectodomain that mediates ligand binding, and a cytosolic TIR domain [132]. In some cases, TLR ectodomains have been shown to bind directly to their ligands [133,134]. By contrast, the affinity of TLR4 for LPS alone is weak and binding is mediated by a soluble accessory protein MD-2 [135,136]. Whether direct or indirect, TLR-ligand binding induces oligomerization (dimerization) of TLR ectodomains, and this initiates signaling through the interaction of cytosolic TIR domains [76,86,89,137]. TIR domains are comprised of 5 parallel beta sheets surrounded by 5 alpha-helices, and mediate homotypic protein-protein interactions in TLR and IL-1R signaling. The signaling enzymes downstream of TLRs do not contain TIR domains, and are thus incapable of interacting directly with TLRs. Instead, they rely on signaling adaptors to bridge the TIR domain of the TLR to the protein-protein interaction domains of signaling enzymes (Figure 1.3).

The most well studied of these signaling adaptors is myeloid differentiation 88 (MyD88), which is used by all TLRs save TLR3 [91,138-140]. MyD88 is relatively simple, consisting of a C-terminal TIR domain and N-terminal death domain (DD). Death domains are found in numerous proteins involved in apoptotic signaling, but also in the IL-1R-associated kinases (IRAKs), which are involved in TLR and IL-1R signaling downstream of MyD88. The DD of MyD88 interacts with the DD of IRAK4, which in turn associates with IRAK2 in a large multimeric helical complex dubbed the myddosome [141]. Myddosome assembly brings IRAKs into close proximity, which initiates auto-phosphorylation of the IRAKs, which in turn phosphorylate and activate TNF receptor associated factor 6 (TRAF6), an

Figure 1.3

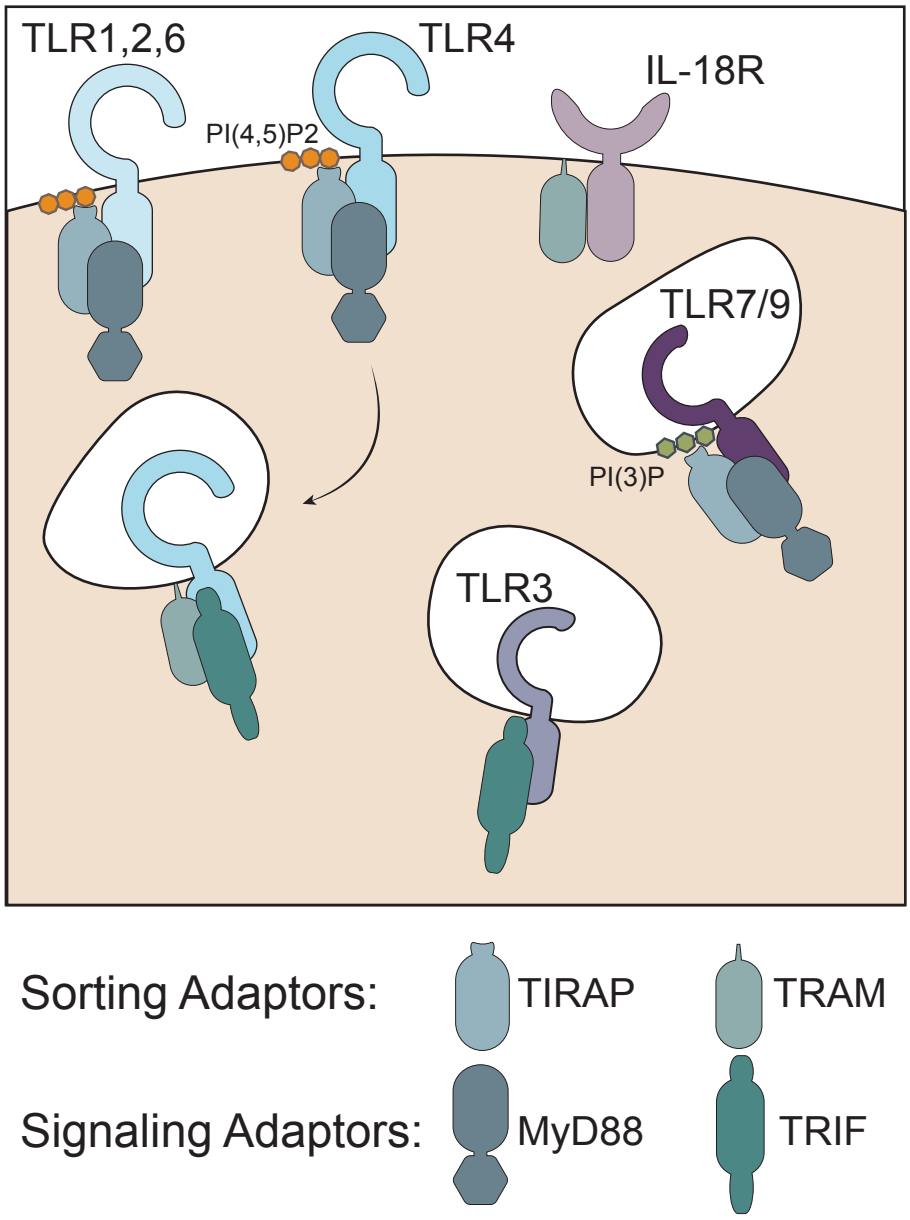

Figure 1.3 TLRs use sorting and signaling adaptors to couple receptor activation to intracellular signaling
See text for explanation.

E3 ubiquitin ligase. TRAF6 builds a platform of K63-linked ubiquitin, which serves as a docking site for a number of other enzymes and related factors that ultimately activate the transcription factors NFκB and AP-1.

MyD88-dependent TLR signaling is implicated in alternative pathways as well. As mentioned in Chapter 1.2, plasmacytoid dendritic cells are capable of activating a signaling pathway that culminates in the activation of IRF7 and the production of type-1 IFN [76,77]. This activity is related but distinct from activation of NFκB and proinflammatory cytokine production in these cells. While IRAK4 is required for activation of both transcription factors, IRAK1 is uniquely required for IRF7 activation and IRAK2 is uniquely required for NFκB activation, suggesting that there may be functionally distinct myddosomes [142,143]. Indeed, IRAK1 knockout mice show enhanced inflammatory cytokine production from pDCs, while IRAK2 knockouts produce more type-1 IFN, suggesting that these enzymes may be competing for upstream activating signals.

MyD88 is also required for signaling downstream of several other receptors. IL-1 and IL-18 receptors also trigger MyD88-dependent signaling pathways requiring IRAK4, IRAK1 and TRAF6, which activate NFκB [144,145]. Further, MyD88 interacts with IFNγR and activates a MAPK-dependent stabilization of some IFNγ-induced transcripts [146]. Though MyD88 can act downstream of TIR-domain containing receptors to stabilize transcripts containing AU-rich elements (AREs) [147], only the death domain of MyD88 is required for this activity downstream of IFNγR, suggesting that some other method of activation is at work. Finally, MyD88 is involved in tumorigenesis in the gut, as MyD88 knockouts are

resistant to tumor formation, though whether this can be attributed to MyD88's known functions or some novel pathway is unknown [148].

The other TLR signaling adaptor is TIR domain containing adaptor protein inducing interferon (TRIF), but this adaptor is only used downstream of TLR3 and TLR4[133,149]. TRIF-dependent signaling events have been less well studied than those downstream of MyD88, but are capable of activating both IRF3 and the production of type-1 IFN as well as NFκB. Studies of TLR4, which is unique in its utilization of both MyD88 and TRIF, have shown that NFκB activation by both signaling pathways occurs in overlapping oscillations, providing prolonged activation.

Sorting Adaptors

Though the TIR of MyD88 is capable of interacting with the TIR of its upstream receptors, yeast 2-hybrid analysis suggests that the affinity of this interaction is quite low [150,151]. In addition to their signaling adaptors, TLRs use sorting adaptors, so called because they are hard-wired to the site of signal transduction. In fact, these adaptors define the site of signal transduction, as they serve to couple ligand-bound receptors to their signaling machinery. Two such sorting adaptors have been defined for toll-like receptors. TIR-containing adaptor protein (TIRAP) has been shown to act downstream of TLR1/2, TLR2/6 and TLR4, linking these receptors to MyD88 [152,153]. TIRAP is a peripheral membrane protein consisting of a C-terminal TIR domain, with which it associates with the TIR domain of TLRs and of MyD88, and an N-terminal localization domain [154,155].

All of the TLRs initially described to require TIRAP are localized to the plasma membrane (See Chapter 1.5), and as initially described, TIRAP's 85 amino acid N-terminus contains a 20 amino acid lipid binding motif containing four crucial lysines that mediate binding to phosphoinositol-4,5-bis-phosphate (PI(4,5)P2) [154]. This phospholipid is enriched at the plasma membrane, specifically at membrane ruffles and sites of endocytosis. The only plasma membrane-localized TLR whose usage of TIRAP is in doubt is TLR5, though recent evidence suggests that TIRAP is likely used downstream of this receptor as well, though the signaling adaptor TRIF has also been implicated [156,157].

While TLR4 uses TIRAP to induce a MyD88-dependent signaling cascade at the cell surface, upon binding to LPS, TLR4 undergoes a CD14-dependent internalization and initiation of an additional TRIF-dependent signaling cascade from endosomes [158,159]. It is in this signaling cascade that TRIF-related adaptor molecule (TRAM) is used. TRAM may be localized to both the cell surface and within endosomes, and contains a bipartite localization domain at its N-terminus, with a required myristolation motif and a polybasic domain that mediates association with the plasma membrane. To date, no plasma-membrane associated function has been ascribed to TRAM in the context of TLR signaling, and indeed a mutant allele of TRAM that cannot associate with the cell surface shows enhanced TRIF-dependent signaling over its wild-type counterpart. However, recent evidence suggests that TRAM may function as a sorting adaptor for IL-18 dependent signaling which is generally thought to occur from the cell surface, though the effects of localization of the adaptor were not directly assessed [160].

Until recently, no sorting adaptor for endosomal TLRs had been described. Aside from TLR4, which signals through both MyD88- and TRIF-dependent pathways, TLR3 is the only endosomal TLR to utilize TRIF, but TLR3 does not require the paired sorting adaptor TRAM. This is not surprising, as the affinity of TRIF's TIR domain for the TIR domain of TLR3 is quite high [150,151]. Though the other endosomal TLRs signal via MyD88, like the cell surface TLRs, they have low affinity for its TIR domain, suggesting that an undefined sorting adaptor may exist. Experiments in TIRAP knockout animals initially excluded TIRAP as a candidate, since cytokine expression downstream of these receptors was not impaired. However, we recently showed that TIRAP is in fact required for endosomal TLR signaling, and that this fact was not apparent because of the use of artificial, non-degradable ligands for those studies (Chapter 2 and Ref 65).

Negative Regulatory Mechanisms

Inflammation may be deleterious to tissues, so preventing or reversing the effects of TLR signaling within cells are necessary. Indeed, negative regulatory mechanisms interfere with the biochemistry of TLR signaling at all stages of the pathway. Of note, several of these negative regulatory mechanisms interfere with the function of the adaptor proteins. For example, a splice variant of MyD88 (MyD88s), which lacks the flexible region connecting the TIR and DD, is upregulated by TLR signaling and acts as a dominant negative, blocking efficient recruitment of IRAK4 [161,162]. Sterile-alpha and armadillo motif-containing protein (SARM) is a TIR-domain containing adaptor protein that seems to interfere with TRIF-mediated signaling in a similar way [163].

The sorting adaptor TIRAP is also a target of negative regulation by cells of the innate immune system. Burton's tyrosine kinase (Btk) was shown to phosphorylate TIRAP downstream of TLR4 and TLR2 activation, and this phosphorylation is thought to recruit suppressor of cytokine signaling 1 (SOCS1) [164]. SOCS1 is then believed to ubiquitinylate TIRAP, targeting it for proteasomal degradation. The protease caspase-1 has also been shown to cleave TIRAP in response to signaling, though it remains unclear whether or not this cleavage is inhibitory [165].

Inflammation is also harmful to infectious microbes, and many pathogens take advantage of the central role of TLR signaling in driving inflammation by targeting several nodes in the signaling pathway. The adaptor proteins in particular are tempting targets, since they mediate the most upstream signaling events. For example, the immediate early herpes simplex virus (HSV) protein ICP0 inhibits TLR signaling by targeting TIRAP for degradationvan [166]. Bacteria also target TLR adaptors, including some that encode their own TIR-domain containing proteins. For example, like TIRAP, TcpB from *Brucella* is a phosphoinositide-binding protein that localizes to the plasma membrane and contains a TIR-like domain [167]. This protein interacts with MyD88 and inhibits NFκB activation downstream of TLRs, though the precise mechanism is unknown [168].

1.5 TLR Cell Biology

Lipid bilayers segregate the exterior environment of a cell from the cytosolic contents. The ligand binding ectodomains of all TLRs face the extracellular space, but it has become increasingly clear that subcellular localization of TLRs is critically important for

their functioning and regulation. The most apparent segregation is between those receptors that reside and encounter ligands at the plasma membrane, and those that are primarily localized to endosomal compartments, but this distinction only tells part of the story, as more recent evidence suggests functionally distinct localization within these categories, as well as ligand-dependent movement that is critical for proper signaling.

Biosynthetic Pathway

TLRs are translocated into the endoplasmic reticulum (ER) during translation and exported to their ultimate destination. TLRs that reside at the cell surface are synthesized in an active form, though they are unlikely to encounter their ligands until reaching their final destination at the plasma membrane. Additionally, since their ligands are predominantly structural proteins unique to microorganisms, encounters with their ligands early in the biosynthetic pathway would still be indicative of infection, and signaling from other locations, if possible, would not be deleterious.

By contrast, endosomal TLRs primarily detect nucleic acid ligands, which are abundant in non-pathological contexts [169]. Though the nucleic acid structures that optimally stimulate these receptors are unique to microbes (as with unmethylated CpG DNA motifs detected by TLR9), many of these receptors are capable of responding to endogenous ligands if present in the right context [170]. For this reason, endosomal TLRs require an additional biosynthetic step to acquire activity, namely cleavage of the ectodomain in lysosomes[171,172]. Restricting active endosomal TLRs to the endocytic environment decreases the potential for autoreactivity, as self-nucleic acids are readily

degraded in lysosomes. Indeed, ectopic expression of TLR9 at the cell surface allows macrophages to respond to self DNA that is typically not stimulatory [173].

All TLRs require chaperones to mediate delivery from the ER. Though some factors in the secretory pathway are likely shared by other transmembrane proteins, several are used more narrowly by TLRs. For example, the ER-resident chaperone called gp96 is responsible for folding both cell-surface (TLR2 and 4) and endosomal TLRs (TLR9), as well as several members of the integrin family of receptors, but not other plasma membrane proteins. Indeed, cells deficient for gp96 are unresponsive to all TLR ligands, suggesting a critical role for all TLRs. TLR4 additionally requires protein associated with TLR4 (PRAT4A) in order to reach the cell surface [174]. Despite its name, PRAT4A seems to be required for trafficking or folding more broadly, since deficient cells are unresponsive to ligands for all TLRs save TLR3 [175].

The most well studied factor regulating TLR trafficking specifically is a multipass transmembrane chaperone called Unc93B1, which is required for ER export for all endosomally-localized TLRs (Figure 1.4) [176,177]. In the absence of Unc93B1, endosomal TLRs fail to load into COP-II coated vesicles, and are thus retained in the ER[178]. This is true for all endosomal TLRs that have been investigated, but distinct regions of Unc93B1 control differential post-golgi trafficking patterns. For example, the C-terminus of Unc93B1 is required to bind to adaptor protein 2 (AP-2), and that loss of this region causes an accumulation of golgi-modified, but uncleaved TLR9. AP-2 facilitates clathrin-mediated endocytosis from the plasma membrane, suggesting that TLR9 is delivered to the cell surface and then endocytosed and delivered to lysosomes [179]. Consistent with

Figure 1.4

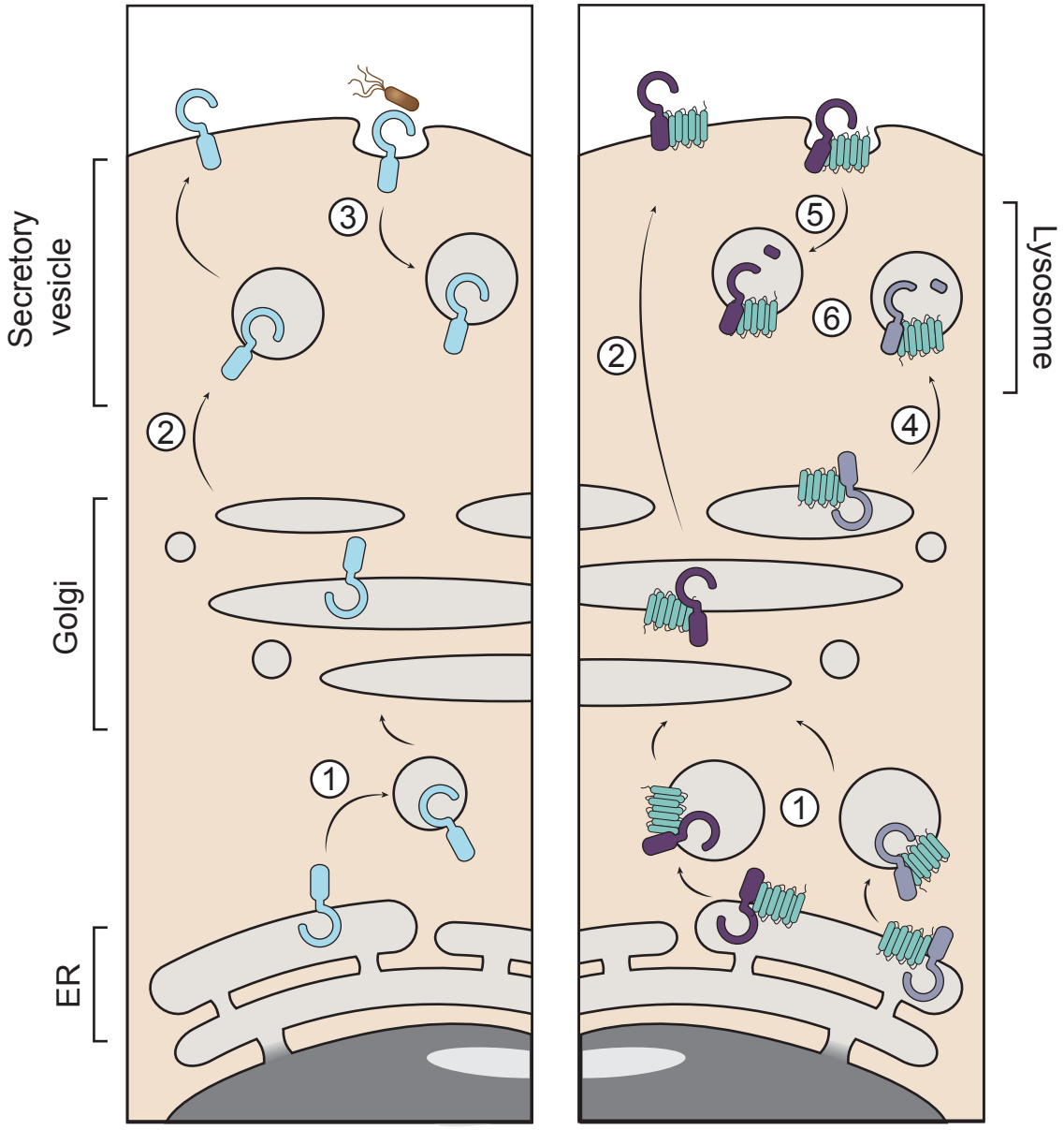

Figure 1.4 The biosynthetic pathway of TLRs
(1) TLRs are synthesized in the ER and loaded into COPII-coated vesicles, dependent on gp96 and other chaperones. TLRs destined for endosomes (right) require Unc93B1.
(2) After glycosylation in the golgi, some TLRs are exported to the cell surface
(3) Upon ligand binding, TLR4 undergoes CD14-dependent endocytosis
(4) TLR7 translocates directly from the golgi to lysosomes through its interaction with AP-4
(5) TLR9 is translocated to the cell surface by Unc93B1. At the cell surface, Unc93B1 interacts with AP-2, which mediates endocytosis and translocation to lysosomes
(6) Cleavage by lysosomal proteases is required for activation of TLR7 and 9.

this hypothesis, mutations in the YxxΦ motif in the c-terminus of Unc93B1 that abolished binding to AP-2 caused accumulation of TLR9 at the cell surface.

Interestingly, though TLR7 also requires Unc93B1 for delivery to lysosomes, abrogating the interaction with AP-2 did not interfere with TLR7 trafficking. Instead, TLR7 binds directly to a different adaptor protein (AP-4), which appears to deliver TLR7 (and TLR11-13) directly to endolysosomes [178].

Localization Post-Synthesis

I previously described how TLRs may reside at the cell surface or within endosomes, but these broad characterizations do not encompass the full complexity of TLR localization. For example, TLR4 is localized to the cell surface at steady state, but is not capable of signaling from any location on the cell surface [154]. As TIRAP is enriched on subdomains of the plasma membrane rich in PI(4,5)P2 TLR4 must be present in these locations in order to effectively induce myddosome formation. Further, even if TIRAP is artificially localized to other domains of the plasma membrane, this is not sufficient to allow signaling through TLR4 [65,154]. This suggests that TLR4 is preferentially localized to PI(4,5)P2-rich regions or rapidly recruited to those regions upon ligand binding. Consistent with the latter hypothesis, TLR4 is rapidly recruited to lipid rafts upon ligand engagement by CD14 [159,180]. One explanation for this signal-inducible movement is that it is an additional regulatory mechanism to ensure fidelity of signaling, as spurious receptor dimerization cannot lead to activation in the absence of intracellular signaling pathways. Though it is clear that signaling must occur from these regions, it is not clear whether exclusion from PI(4,5)P2 prior to ligand engagement has a regulatory purpose.

The best understood example of ligand-dependent movement also involves TLR4. Following ligand engagement at the cell surface and activation of the MyD88-dependent signaling pathway, TLR4 translocates to an endosomal compartment, whereupon it engages the TRIF-dependent signaling pathway [158]. This process is also CD14-dependent, and cells lacking CD14 lack the ability to activate TRIF signaling and type-1 IFN production when encountering soluble ligand [159]. This is true both for genetically deficient cells as well as cells that naturally lack expression of CD14, such as B-cells. Interestingly, TLR4 ligands on particles that can be phagocytosed in the absence of CD14 are capable of activating the TRIF pathway. Since B-cells typically restrict phagocytosis to particles that are bound by their clonal receptor, this may provide a useful regulatory mechanism whereby only B-cells specific for a particular antigen will be capable of triggering the TRIF pathway.

Whether other surface TLRs undergo similar trafficking and signaling remains controversial. Inflammatory monocytes produce type-1 IFN in a TLR2-dependent manner in response to vaccinia virus and MCMV [181]. As with TLR4, this IFN production is dependent on internalization, but relies on MyD88- and IRF7-dependent signaling rather than TRIF. This is similar to the MyD88-dependent IFN production in response to nucleic acid ligands in pDCs, indicating that multiple cell types have non-canonical specialization of TLR pathways [76,182]. Though some investigators have reported TLR2 internalization and IFN production in macrophages in response to particular bacterial ligands [183-185], these data have been disputed[186] and we cannot reproduce these results in our lab. In any case, it is interesting to note that type-I IFN-producing signals never

originate at the plasma membrane, suggesting that internalization of receptors is an important regulatory step to preclude spurious induction.

In pDCs, NFκB activation and IRF7-dependent IFN production appear to occur from different locations, as these signaling pathways can be dissociated by deletion of the adaptor protein AP-3; pDCs from mice lacking AP-3 fail to produce type-1 IFN, but have increased production of inflammatory cytokines [78]. Interestingly, though macrophages do not usually produce type-1 IFN in response to TLR9 ligands, delivering CpG-DNA with the transfection reagent DOTAP causes macrophages to activate the IRF7 pathway in an AP-3-dependent manner. The precise nature of these endosomes remains unclear, though the fact that delivery of ligand by transfection reagent is enough to rescue IRF7 activation in macrophages might indicate that trafficking of TLR9 to IRF7 endosomes is independent of ligand.

On the one hand, microscopic analysis suggests that stimulation with DOTAP-CpG induces AP-3-dependent migration from VAMP3+ early endosomes to LAMP2+ Lysosome-related organelles (LROs), suggesting that this movement is at least partially controlled by TLR signaling. On the other hand, the initial characterization of the effect of DOTAP on CpG trafficking shows that in the absence of DOTAP, IFN-inducing CpG collects in late endosomal compartments, while being distributed more evenly throughout the endosomal network in the presence of DOTAP[187]. These data are consistent with IFN production occurring from early endosomal compartments, and TLR9 may typically pass through these compartments too quickly in macrophages.

It is interesting to note that targeting TRAF3, an enzyme required for the IFN response downstream of TLR9, to endosomes rich in 3' phosphoinositides is sufficient to rescue the signaling defects of AP-3 knockout macrophages [78]. This may suggest that the role of AP-3 is to deliver certain cytosolic signaling factors like TRAF3 to TLR9-containing endosomes, though these same data would also be consistent with delivery of TLR9 to endosomes that typically associate with TRAF3. In either case, this experiment suggests that IFN-producing endosomes are defined by association with some cytosolic factor(s), rather than the lumenal components of the endosome. It is possible that some as-yet unidentified sorting adaptors are responsible for assembling the needed signaling components differentially between these two different endosomes.

1.6 Conclusion

Only 25 years have passed since Charles Janeway first proposed pattern recognition receptors as a theoretical framework for understanding innate immune recognition, and less than 20 have passed since the first of these receptors was identified [1,12]. Because this field has grown up in the modern genetic era, the trajectory of discovery for the important components of TLRs and other PRRs has followed a consistent pattern. Investigators used forward genetic analysis or large unbiased screens to identify molecules of interest, followed quickly by scouring the mammalian genome for related factors to investigate via reverse genetics. These approaches have been wildly successful, but I believe it is safe to echo Janeway's pronouncement on the fate of adaptive immune recognition in 1989 and say that our understanding of the genetics of innate immune recognition is approaching an asymptote.

Yet many exciting avenues of investigation remain. Subcellular localization of TLRs is clearly critically important to their function, yet our understanding is limited to a handful of receptors and a handful of cell types. Biochemical analysis of these pathways has been largely restricted to complementing genetic knockouts through ectopic expression. Since this approach often leads to spontaneous signaling, many questions regarding kinetics and ligand-dependency remain unresolved. Finally, most investigations of TLR signaling to date have relied on synthetic microbial ligands that ignore the full context of infection. Since many microbes contain many PAMPs, this approach has been useful to control for the actions of multiple separate pathways, but as described in the following chapter, it may also obscure important features of these pathways.

Chapter 2: A Promiscuous Lipid-binding Protein Diversifies the Subcellular Sites of Toll-like Receptor Signal Transduction

2.1 Abstract

The Toll-like receptors (TLRs) of the innate immune system are unusual in that individual family members are located on different organelles, yet most activate a common signaling pathway important for host defense. It remains unclear how this common signaling pathway can be activated from multiple subcellular locations. Here, we report that, in response to natural activators of innate immunity, the sorting adaptor TIRAP regulates TLR signaling from the plasma membrane and endosomes. TLR signaling from both locations triggers the TIRAP-dependent assembly of the myddosome, a protein complex that controls proinflammatory cytokine expression. The actions of TIRAP depend on the promiscuity of its phosphoinositide-binding domain. Different lipid targets of this domain direct TIRAP to different organelles, allowing it to survey multiple compartments for the presence of activated TLRs. These data establish how promiscuity, rather than specificity, can be a beneficial means of diversifying the subcellular sites of innate immune signal transduction.

2.2 Introduction

At some point in their existence, all proteins must move within mammalian cells. Some movement is biosynthetic, in that the protein must be transported from its site of synthesis on the ribosome to its site of action. Another type of movement is signal-dependent, meaning that the protein will move from one location to another in response to some cellular stimulus. Classic cell biological studies identified the cis-acting

sequences that direct these types of protein movement within cells; for example[188,189,190]. In addition to these examples, a more complex type of protein movement exists, where proteins can be found in multiple locations under resting conditions, or can be recruited to multiple locations in response to a stimulus. In this latter instance, it is not obvious how a single localization signal would allow a protein to be targeted to (and function from) multiple organelles. An example of this can be found from the studies of the Toll-like Receptors (TLRs) of the innate immune system.

TLRs are transmembrane receptors that are expressed by a variety of mammalian cell types, but are best studied in professional phagocytes such as macrophages and dendritic cells (DCs) [191]. TLRs detect a wide range of microbial products, and may be divided into different groups based on their subcellular site of ligand recognition [91]. TLRs 1, 2 and 4-6 reside at the plasma membrane, where they detect molecules displayed on the surface of various pathogens. TLRs 3, 7-9 and 11-13 are localized to various endosomal compartments, where most detect microbial nucleic acids.

Despite residing in distinct subcellular compartments, most TLRs activate a common signal transduction pathway to induce innate and adaptive immunity. TLR signaling usually initiates with the activation of an adaptor protein called MyD88, which is recruited to the conserved TIR domain present in the cytosolic tail of all receptors of this family [137,192]. MyD88 forms a protein complex with kinases of the IRAK family called the myddosome [141,193]. This complex is thought to induce a cascade of signaling events that activates the NF-κB-dependent expression of cytokines, chemokines and other immunomodulatory factors [137,193,194]. Because TLRs reside on distinct organelles, the

myddosome must have the capacity to be assembled in multiple subcellular locations. How myddosome assembly can be promoted from multiple locations is unknown. Answering this question will fill a fundamental gap in our understanding of how immune signaling pathways are integrated into the cellular infrastructure within which they operate.

A simple explanation for how MyD88 can be recruited to diverse organelles would be through interactions with the TIR domains of activated TLRs. However, 2-hybrid analyses performed in yeast and mammalian cells indicated that MyD88 has limited ability to interact with TLRs directly [150,151]. For this reason, an intermediate protein is likely required to link activated TLRs to the recruitment of MyD88. Consistent with this model, the cell surface TLRs utilize “sorting adaptors” to accomplish this task. Sorting adaptors are the only regulators of TLR signaling that are located at the subcellular site of signal transduction, prior to any microbial encounter [169]. Their placement at the eventual site of signaling allows sorting adaptors to function as sensors of activated TLRs, and recruit downstream signaling adaptors (*e.g.* MyD88) to induce inflammatory cytokine expression. Most plasma membrane-localized TLRs use the sorting adaptor TIRAP (also known as Mal) to recruit MyD88 to the cell surface [152,154,195,196]. The ability of TIRAP to function as a sorting adaptor is dependent on its amino terminal localization domain, which interacts with plasma membrane-localized phosphatidylinositol-4,5 bisphosphate (PI(4,5)P2) and other lipids [154]. The use of sorting adaptors extends beyond the MyD88-dependent pathways, as analogous systems exist in other immune signaling pathways in mammals and *Drosophila melanogaster* [158,169,197].

Despite the apparent importance of sorting/signaling adaptor pairs for controlling TLR signaling from the cell surface, a sorting adaptor for the exclusively endosomal TLRs has not been described. As such, it is unclear how MyD88-dependent innate immune responses are activated by endosomal TLRs. TIRAP was initially excluded as a sorting adaptor for endosomal TLRs because TIRAP-deficient cells retain the ability to respond to synthetic TLR7 and TLR9 ligands [152,196]. However, these studies also demonstrated that the requirement of TIRAP for signaling from cell surface-localized TLRs can be bypassed when high concentrations of ligand are used. Primary macrophages and DCs are highly endocytic. This property, combined with the common use of phosphorothioate-linked (nuclease-resistant) nucleic acids to activate endosomal TLRs, led us to hypothesize that these ligands could accumulate to high concentrations within endosomes, masking a requirement for TIRAP. In light of this hypothesis, we decided to reassess the role of TIRAP in endosomal TLR signaling.

Herein we report that TIRAP is required for signaling downstream of endosomal TLRs in response to natural ligands, such as viral nucleic acids. We show that TIRAP is present in myddosomes induced by both cell surface and endosomal TLRs, and is required for myddosome formation. Further, we show that the ability of TIRAP to bind to multiple lipid species is critical for its ability to function from multiple subcellular compartments. These findings provide a molecular explanation for the ability of MyD88 to be recruited to more than one organelle, and highlight how a promiscuous lipid-binding domain can be used to diversify the subcellular sites of innate immune signal transduction.

2.3 Results

TIRAP is required for endosomal TLR signaling in response to viral infection

To determine if TIRAP would function as a sorting adaptor for endosomal TLRs, we readdressed the genetic requirement for this protein in the signaling pathway activated by TLR9. TLR9 is an excellent model for these studies for two reasons. First, it is the best-characterized endosome-localized TLR [198] and its ligand (unmethylated CpG-containing DNA) is easy to produce [199]. Second, whereas several viruses activate TLRs at the cell surface and endosomes [92,181,200], substrains of herpes simplex virus (HSV) exist that only activate TLR9 [93]. Thus, TLR9 provides a useful model for the study of both synthetic (CpG DNA) and natural (HSV) activators of endosomal TLRs.

Wild type (WT) and TIRAP-knockout (KO) primary bone-marrow derived macrophages (BMDM) were stimulated with CpG DNA or three substrains of HSV-1 [93]. The cell populations were then assessed for the expression of the cytokines interleukin-1β (IL-1β) and IL-6. As expected [152,196], TIRAP KO BMDM had no defect in responding to CpG DNA, while their response to bacterial lipopolysaccharide (LPS), a ligand for the cell-surface TLR4, was impaired (Figure 2.1A). Interestingly, whereas all three substrains of HSV induced the expression of IL-1β and IL-6 in WT BMDM, TIRAP KO BMDM were defective for these responses (Figure 2.1B). Among the HSV substrains tested, Kos A and Kos CE only engage TLR9, whereas Kos K engages both cell surface TLR2 and endosomal TLR9 [93]. Our finding that TIRAP is required for cytokine expression in response to HSV substrains that only engage TLR9 suggests that this adaptor plays a role in signaling from endosomal receptors. Interestingly, as was observed for ligands

Figure 2.1

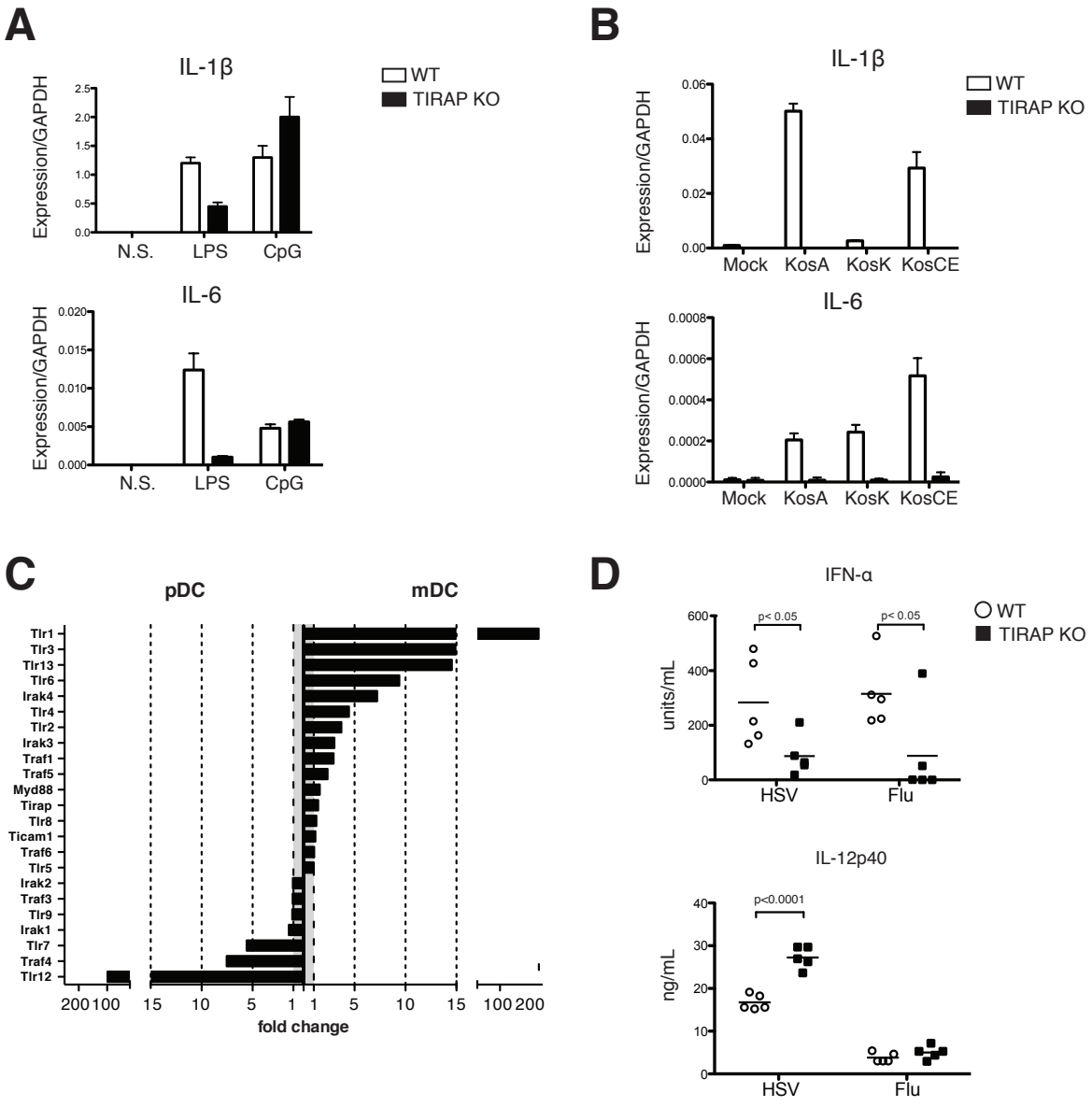

Figure 2.1 TIRAP Is Required for Endosomal TLR Signaling in Response to Natural Ligands
(A and B) Primary WT or TIRAP KO BMDMs were treated with 100 ng/ml LPS or 1 mM CpG DNA (A) or infected with HSV (B) for 3 hr. Total mRNA was extracted and analyzed for expression of IL-1b and IL-6. Note that TIRAP KO BMDMs are defective in responding to natural ligands. N.S., cells that were not stimulated.
(C) Microarray gene expression profiles from sorted cDCs and pDCs were analyzed for transcripts associated with TLR signaling pathways. Values on the x axis represent the fold difference in gene expression between mDCs and pDCs. Gray area depicts equal gene expression in cDCs and pDCs. Note that TIRAP is expressed in pDCs comparably to cDCs, despite the absence of cell-surface TLRs.
(D) pDCs from WT or TIRAP KO mice were infected with HSV-2 (186 syn+) at an moi of 1 or influenza (A/PR/8) at an moi of 10. Supernatants were collected after 24 hr and analyzed by ELISA. Error bars represent SD. See also Figure S1.

that activate cell surface-localized TLRs [152], the requirement for TIRAP in TLR9 signaling can be bypassed by increasing the dose of a subset of HSV substrains (Supplementary Figure S1A, MOI=10). The requirement of TIRAP for antiviral responses was unique to the TLR pathway, as WT and TIRAP KO BMDM expressed the interferon (IFN) inducible gene viperin comparably when infected with mammalian reovirus (Supplementary Figure S1B), a known activator of the cytosolic RIG-I like receptors [62]. Collectively, these data suggest an important role of TIRAP in the response to natural (viral) activators of endosomal TLR9.

To corroborate these findings, we sought an alternative cell type to study the role of TIRAP. Plasmacytoid dendritic cells (pDCs) are an intriguing option, as they exclusively utilize endosomal TLRs to detect infections [201]. As such, all of the plasma membrane-localized TLRs that are established to require TIRAP are non-functional in these cells [93]. Moreover, in pDCs, endosomal TLRs exhibit an intriguing behavior in that they can induce cytokine and IFN production from different populations of endosomes, with IFN production occurring from endosomes rich in 3' phosphoinositides [78]. We reasoned that if TIRAP was not required for endosomal TLR signaling, as previously believed [152,196], then pDCs would have no need to express the gene encoding this adaptor. To address this possibility, a comparison of gene expression was performed between highly purified pDCs and conventional DCs (cDCs). This analysis revealed that pDCs express much lower levels of the cell-surface TLRs 1, 2, 4 and 6 than cDCs (Figure 2.1C). This observation may (in part) explain the inability of pDCs to respond to ligands for plasma membrane-localized TLRs [93]. pDCs expressed higher levels of some endosomal TLRs (TLR7 and TLR12) than their cDC counterparts. Interestingly, both cell types expressed

comparable levels of TLR9, TIRAP and MyD88 (Figure 2.1C), suggesting that TIRAP may indeed have a function downstream of endosomal TLRs in pDCs. To address this possibility, pDCs were infected with either HSV or influenza virus, natural activators of endosomal TLR9 and TLR7, respectively [74,96]. Infected cells were then assessed for their ability to induce the expression of IFNα or the cytokine IL-12p40. Because the expression of these cytokines is initiated from different populations of endosomes [78], this experiment allows us to determine if a population of endosomes exists that selectively requires TIRAP function. In comparison to WT pDCs, TIRAP KO pDCs were impaired in the ability to produce IFNα in response to HSV or influenza virus (Figure 2.1D). In contrast, the production of IL-12p40 was not impaired in TIRAP KO pDCs, suggesting that TIRAP regulates signaling from endosomes that were previously defined as being rich for 3' phosphoinositides [78]. Another sorting adaptor may exist to control TLR signaling to induce IL-12p40. Overall, these data establish that, in response to natural activators of innate immunity, TIRAP is required for signaling by endosomal TLRs in multiple cell types. Furthermore, the use of HSV strains that only activate TLR9, and the use of pDCs that only permit signaling by endosomal TLRs, eliminates the possibility of contaminating bacterial products (*e.g.* LPS) explaining these observations.

Immortalized macrophages are a model for investigating endosomal TLR signaling

To define the means by which TIRAP regulates TLR signaling from multiple organelles, we sought a cell type that would be easy to propagate and amenable to genetic manipulation. Immortalized BMDM (iBMDM) have emerged as a useful tool in this regard, as they retain the signaling properties of their primary cell counterparts [62,63] and

TIRAP KO iBMDM have been used to dissect the functions of this adaptor [202]. Similar to our observations made in primary BMDM, TIRAP was required for iBMDM to respond to several substrains of HSV, including two that engage only TLR9 (Kos A and Kos CE) (Figure 2.2A) [93]. We found that iBMDM are less phagocytic than primary BMDM (Figure 2.2B). This was expected, because endocytosis and phagocytosis rates are higher in non-mitotic macrophages (*e.g.* primary BMDM) than in dividing macrophages [203]. We hypothesized that this lower phagocytic activity would prevent CpG DNA from building to high concentrations within endosomes, perhaps revealing a requirement for TIRAP in responses to synthetic TLR9 ligands. Indeed, while WT iBMDM responded to CpG DNA comparably to primary WT BMDM, TIRAP KO iBMDM were completely unresponsive (Figure 2C). However, high doses of CpG DNA partially overcame the requirement for TIRAP in these cells (Figure S1C). To determine if TIRAP deficiency was responsible for the unresponsiveness of these cells to TLR9 ligands, this adaptor was stably reintroduced into TIRAP KO iBMDM via retroviral transduction. The retroviral vector used also encodes an internal ribosomal entry site upstream of eGFP, which allowed for the use of fluorescence activated cell sorting (FACS) to isolate a population of cells with uniform levels of expression (data not shown). Rescuing TIRAP expression in TIRAP KO iBMDM restored responsiveness to plasma membrane and endosomal localized TLR ligands (Figure 2.2D). Furthermore, rescuing TIRAP expression enabled an enhanced response to all substrains of HSV examined (Figures 2.2A and S1D). Among the substrains examined is HSV 7134 (Figure S1D), which lacks the expression of ICP0 [166]. ICP0 is an immune evasion protein that may interfere with TLR signaling by causing the degradation of TIRAP and/or MyD88 [166]. To determine if ICP0 is acting to prevent

Figure 2.2

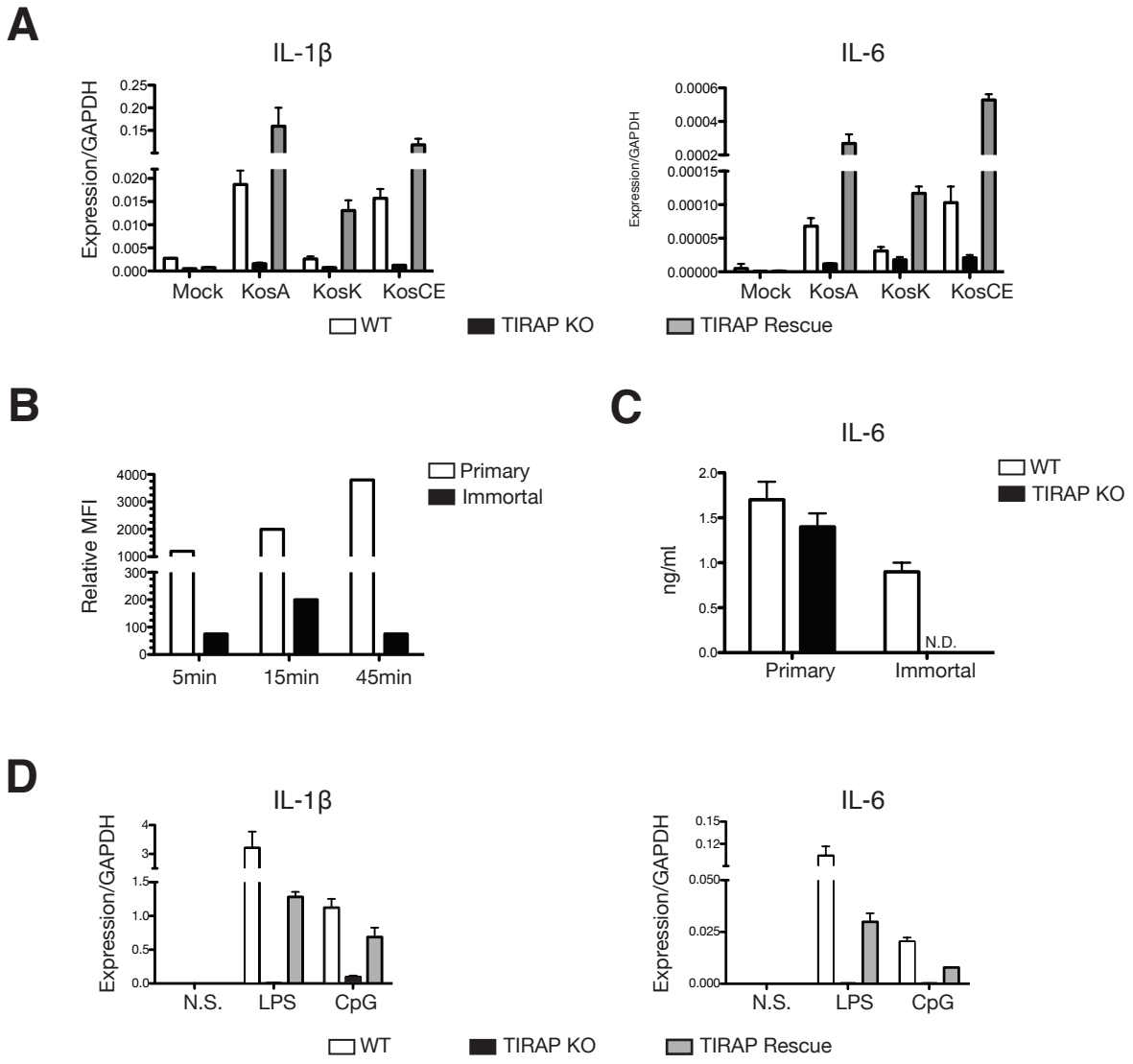

Figure 2.2 iBMDM Responses to CpG DNA Mimic Primary Cell Responses to Natural Ligands
(A) WT, TIRAP KO, or TIRAP-expressing TIRAP KO iBMDMs were infected with indicated HSV strains (moi = 1) for 3 hr and analyzed by qPCR.
(B) Primary or iBMDMs were incubated with fluo- rescent beads at 37□ C for the indicated times, and phagocytosis was analyzed by flow cytometry. Mean fluorescence intensity was plotted relative to cells incubated with beads on ice for 45 min.
(C) WT or TIRAP KO primary and iBMDMs were stimulated with 1mM CpG DNA. After 24 hr, supernatants were collected and analyzed for IL-6 by ELISA. N.D., no signal was detected.
(D) WT, TIRAP KO, or TIRAP-expressing TIRAP KO iBMDMs were treated with LPS or CpG DNA for 3 hr and analyzed by qPCR.
Error bars represent SD. See also Figure S1.

TLR signaling in our assays, side-by-side experiments were performed with HSV 7134 and an isogenic substrain whose expression of ICP0 was restored (7134R). We observed no difference in the ability of these strains to induce TIRAP-dependent cytokine expression (Supplementary Figure S1D). These data suggest that ICP0 may act at a later stage of infection or in a different cell type to interfere with TLR signaling. Overall, the observation that the signaling defects of TIRAP KO cells can be rescued by the expression of a TIRAP cDNA provides formal genetic proof of its role in signal transduction activated by endosomal TLRs. iBMDM therefore provide a genetically tractable model to study the role of TIRAP in signaling from endosomal TLRs through the use of natural and synthetic ligands.

TIRAP promotes the assembly of myddosomes at the plasma membrane and endosomes.

Based on the experiments described above, it was possible that TIRAP acts to assemble myddosomes after activation of TLRs found at the plasma membrane and endosomes. The crystal structure of the myddosome indicates that the adaptor MyD88 is a core component of this signaling complex [141]. However, the natural kinetics of myddosome assembly induced by TLR ligands remain undefined. To assess myddosome assembly, iBMDM were stimulated with LPS or CpG DNA, and endogenous MyD88 immunoprecipitates were subjected to western analysis for the presence of the other known components of the myddosome, the kinases IRAK2 and IRAK4 [141,193]. Within minutes of stimulating cells with either LPS or CpG DNA, both kinases were recruited to MyD88 (Figures 2.3A and 3B). Immunoprecipitations with

Figure 2.3

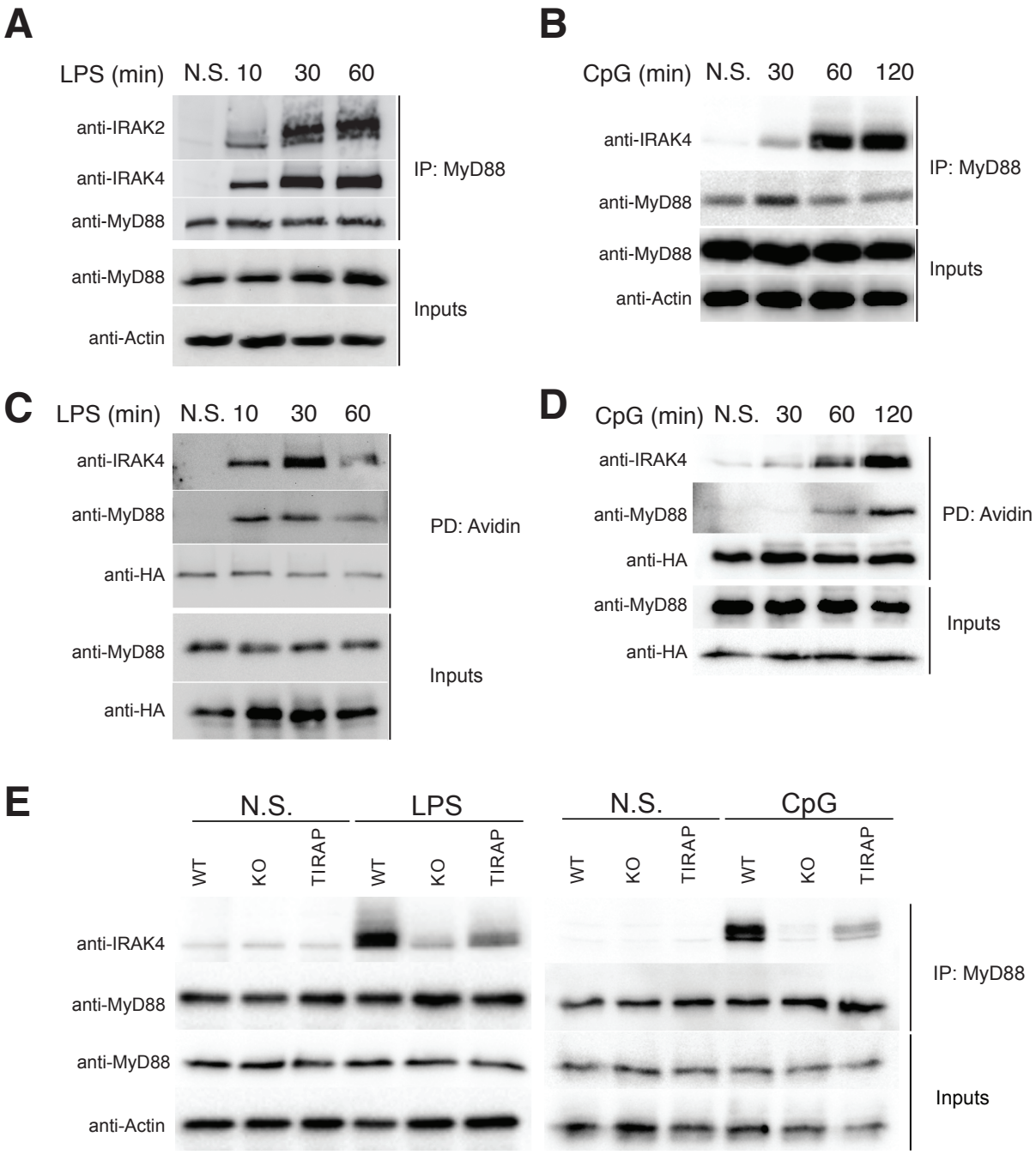

Figure 2.3 TIRAP Is a Critical Constituent of the Myddosome
(A and B) WT iBMDMs were stimulated with LPS (A) or CpG DNA (B) for the indicated times. MyD88 was immunoprecipitated (IP) from lysates and analyzed by western blot.
(C and D) TIRAP-transgenic iBMDMs were stimu- lated with LPS (C) or CpG DNA (D), and biotin- TIRAP was precipitated from cleared lysates with avidin-coated agarose before western analysis. (E) WT, TIRAP KO, or TIRAP-expressing TIRAP KO iBMDMs were treated with LPS for 1 hr or CpG DNA for 2 hr and analyzed as in (A). N.S., cells that were not stimulated.
See also Figure S2.

IRAK2 antisera yielded comparable results, with MyD88 and IRAK4 being recruited to IRAK2 within minutes of stimulation (Figure S2A). Consistent with prior work on the activation of MAP kinases and NF-κB [138,152], LPS induced maximal assembly of the myddosome more rapidly than CpG DNA (Figures 2.3A and 3B). Because the myddosome is not detected in resting cells, its assembly can be used to monitor the earliest cytosolic events that occur during TLR signaling.

To determine if TIRAP is a component of LPS or CpG DNA-induced myddosomes, we utilized the transgenic TIRAP KO cells described above. The TIRAP allele expressed in these cells contains a biotinylation sequence and an HA-epitope tag to facilitate biochemical analysis. LPS and CpG DNA-induced myddosomes containing IRAK4 and MyD88 could be detected by direct isolation of biotinylated TIRAP through the use avidin-coated beads (Figure 2.3C and 3D). Moreover, TIRAP could be recruited to MyD88-immunoprecipitates in LPS treated cells (Figure S2B). These data indicate that TIRAP is a stable component of the myddosomes induced by either TLR4 or TLR9.

To determine if TIRAP was required for myddosome formation, TIRAP KO iBMDM were examined. Myddosome formation in response to both LPS and CpG DNA was impaired in TIRAP KO cells, but rescued in TIRAP KO cells expressing the TIRAP transgene (Figure 2.3E). These data establish that TIRAP controls the assembly of (and is a component of) myddosomes formed in multiple subcellular compartments.

Promiscuous lipid binding diversifies the subcellular sites of TIRAP residence

TIRAP is a peripheral membrane protein that is enriched at the cell surface [204]. Our discovery that TIRAP regulates TLR signaling from endosomes therefore raises the

question of how a plasma membrane-localized adaptor can function from endosomes. The localization of TIRAP is strictly dependent on an amino terminal lipid-binding domain that interacts with acidic phosphoinositides (PIs) and phosphatidylserine (PS) [204]. To confirm these observations, the ability of TIRAP to bind lipids was assessed by PIP-strip analysis, which permits the identification of several possible protein-lipid interactions in a single experiment. GST-TIRAP interacted with all phosphorylated PIs and PS (Figure 2.4A). As a control for specificity of these interactions, we used a mutant that is defective for lipid-binding *in vitro* and localization *in vivo* (TIRAP 4X). This mutant TIRAP allele did not bind to any lipids in this analysis (Figure 2.4A). Among the lipids TIRAP interacts with, PI(4,5)P2 is considered solely important for its function in controlling signaling by plasma membrane-localized TLRs [155,204]. In this regard, TIRAP is similar to many other lipid-binding proteins in that its ability to bind PIs promiscuously is not considered functionally significant [205].

Because some of the lipids that TIRAP interacts with are enriched on endosomes (*e.g.* PI(3)P and PI(3,5)P2) [206], we considered the possibility that a pool of TIRAP would be located on endosomes. This possibility was addressed by examining the localization of the lipid-binding domain of TIRAP in primary BMDM. We transfected these cells with plasmids encoding the GFP-tagged lipid-binding domain of TIRAP (TIRAP-loc) along with a plasmid encoding a cherry-tagged pleckstrin homology (PH) domain from PLCδ1 (PLC), which binds to PI(4,5)P2 uniquely [207]. We reasoned that if TIRAP binds only to PI(4,5)P2 within cells, then the subcellular distribution of these two proteins should be identical. However, if TIRAP binds to more lipids than PI(4,5)P2, then the distributions of these proteins should be overlapping but distinct. Confocal microscopy revealed that

Figure 2.4

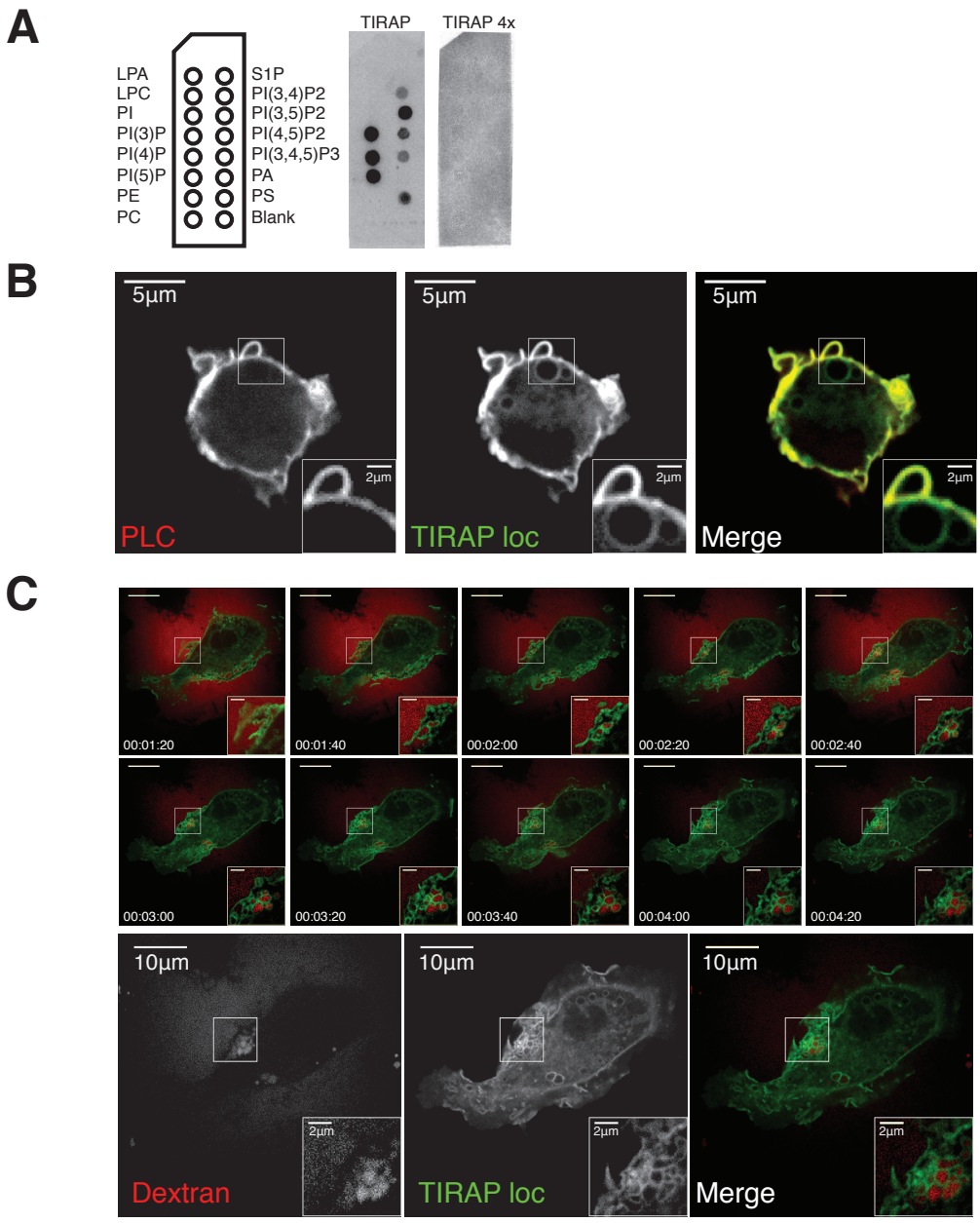

Figure 2.4 Promiscuous Lipid Binding Diversifies the Localization of TIRAP
(A) GST-tagged TIRAP or TIRAP 4X proteins were incubated with PIP strips containing various lipids (shown in left panel) and assessed for protein binding by far western analysis.
(B) TIRAP KO primary BMDMs expressing TIRAP loc-GFP and PLC-cherry were analyzed by confocal microscopy. All images are representative of at least three independent experiments where more than 200 cells were examined per condition and >95% of the cells displayed similar staining. Selective localization of TIRAP to intracellular vesicles compared to the PLCd1 PH domain demonstrates that TIRAP binds to multiple lipids.
(C) TIRAP KO primary BMDMs expressing TIRAP loc-GFP were analyzed by confocal microscopy. One image was captured every 20 s for 20 min. (C) Shows representative frames from one such capture (see Movie S1 for full-length movie).
See also Movie S1.

both proteins colocalized extensively at the plasma membrane, which is an abundant site of PI(4,5)P2 [207]. Interestingly, TIRAP's lipid-binding domain also labeled intracellular vesicles that were devoid of the PLCδ1 PH domain (Figure 2.4B, inset), suggesting that TIRAP binds additional lipid targets inside cells, as it does *in vitro*.

To determine if the intracellular TIRAP-positive compartments were endosomes, live time-lapse microscopy was performed in primary BMDM expressing the GFP-tagged lipid-binding domain of TIRAP. Primary BMDM were highly active and exhibited extensive ruffling of the plasma membrane (Supplementary Movie). Numerous TIRAP positive vesicles were formed at these sites of membrane ruffling, and these vesicles accumulated Alexa-647 labeled dextrans that were added to the culture media shortly after imaging began (Figure 2.4C and S3). These data indicate that the lipid-binding domain of TIRAP is capable of localizing to the plasma membrane and *bona fide* endosomes. Promiscuous lipid binding by TIRAP may therefore be important for its ability to function as a sorting adaptor for TLRs located at the cell surface and endosomes.

Distinct lipid targets of the TIRAP localization domain permit TLR signaling from the plasma membrane and endosomes.

To determine the function of the individual lipids with which TIRAP interacts, the lipid-binding domain of TIRAP was replaced with domains of singular specificity (Figure 2.5A, top panel). We replaced the endogenous lipid-binding domain of TIRAP with the PLCδ1 PH domain described above, which binds exclusively to PI(4,5)P2 (hereafter referred to as PLC-TIRAP). By a similar strategy, a PI(3)P-specific TIRAP allele was generated

Figure 2.5

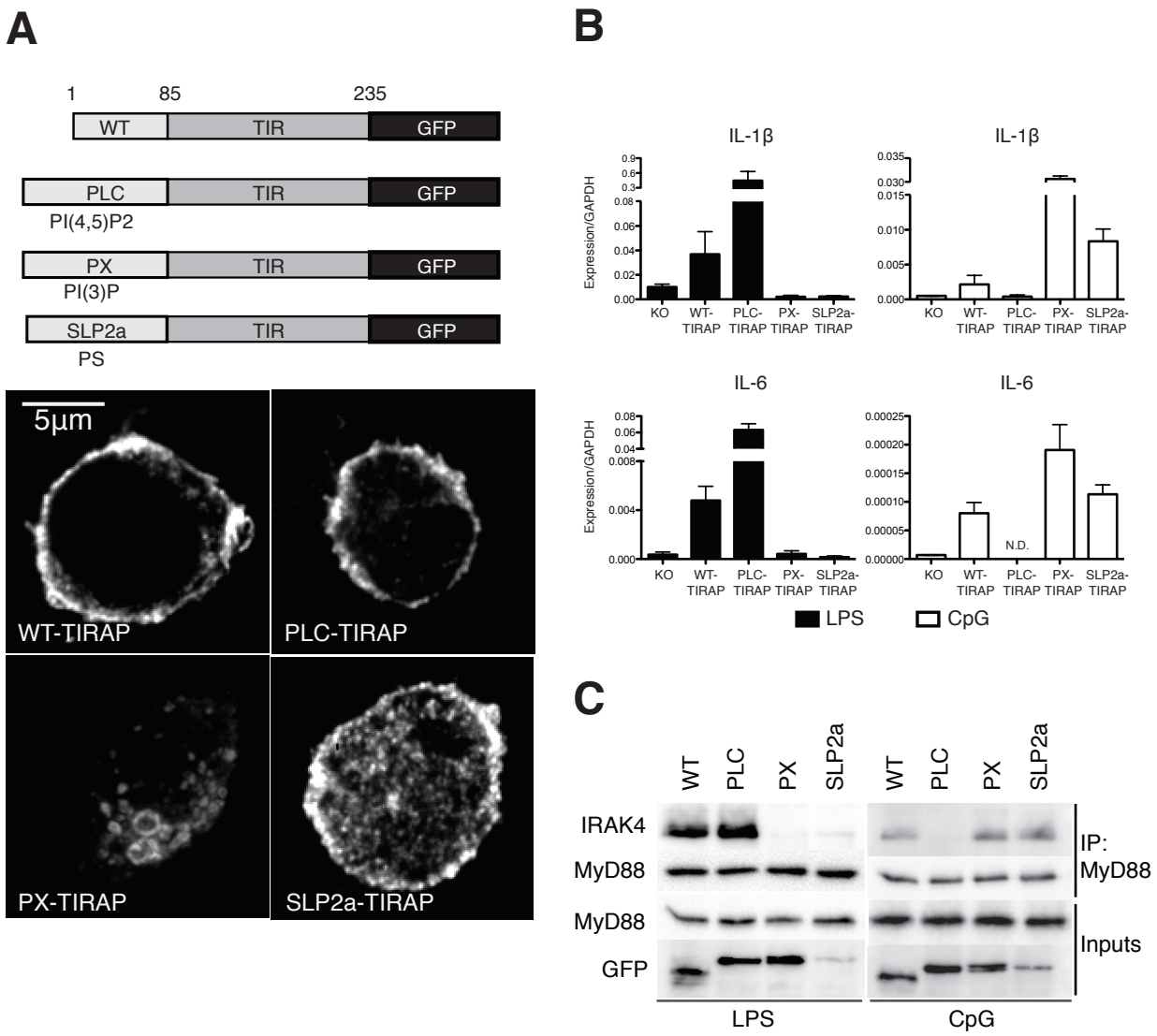

Figure 2.5 Promiscuous Lipid Binding by TIRAP Diversifies the Sites of TLR Signaling
(A) Confocal microscopic analysis of TIRAP KO iBMDMs stably transduced with GFP-tagged TIRAP alleles that contain different lipid-binding domains (depicted in the top panel). The micrographs demonstrate selective localization of TIRAP dependent on its lipid-binding domain. All images are representative of at least three independent experiments where over 200 cells were examined per condition and >95% of the cells displayed similar staining.
(B) Cells from (A) were stimulated with LPS or CpG DNA and analyzed by qPCR. Note that selective binding of TIRAP to distinct lipids permits signaling from distinct compartments.
(C) Cells from (A) were stimulated with LPS or CpG DNA for 1 hr, and myddosome formation was assessed.

called PX-TIRAP. This adaptor has the localization domain of p40-phox, which binds exclusively to PI(3)P on endosomes [93,204]. PX-TIRAP was of particular interest because of our finding that TIRAP preferentially regulates IFNα expression induced by TLR7 and TLR9 (Figure 2.1D), which occurs from endosomes containing 3' phosphoinositides in pDCs [78]. Finally, SLP2a-TIRAP was generated, which binds PS, a general component of the cell surface and endosomal membranes [208]. These constructs were fused to GFP, stably transduced into TIRAP KO iBMDM and sorted by FACS to isolate clones of comparable expression levels. The resulting cell populations were examined by confocal microscopy, which verified their expected localization (Figure 2.5A, bottom panel). PLC-TIRAP was detected primarily at the cell surface, PX-TIRAP was enriched on intracellular vesicles previously identified as endosomes [204], and SLP2a-TIRAP was detected at the cell surface and endosomes.

This set of stable macrophage lines was stimulated with LPS or CpG DNA to determine which lipids were important for TLR signal transduction. As expected, TIRAP KO cells expressing WT TIRAP regained the ability to permit TLR4 signaling from the cell surface and TLR9 signaling from endosomes (Figure 2.5B). The cell surface-exclusive PLC-TIRAP restored responsiveness to the TLR4 ligand LPS, but interestingly, this allele did not restore TLR9 signaling in response to CpG DNA (Figure 2.5B). These data indicate that whereas PI(4,5)P2 binding by TIRAP is sufficient for signaling from the plasma membrane, it is not sufficient for signaling from endosomes. Remarkably, examination of the function of PX-TIRAP yielded the opposite results. TIRAP KO cells expressing PX-TIRAP (localized to endosomes via PI(3)P), responded robustly to CpG DNA but did

not respond to LPS (Figure 2.5B). Similar results were obtained with the PS-binding SLP2a-TIRAP allele (Figure 2.5B).

The localization of TIRAP was also critical for myddosome formation. Consistent with Figure 3B, TIRAP KO cells reconstituted with WT TIRAP formed myddosomes in response to both LPS and CpG DNA (Figure 2.5C). By contrast, PLC-TIRAP was able to rescue myddosome formation solely in response to LPS, while PX-TIRAP and SLP2a-TIRAP were able to rescue myddosome formation in response to CpG DNA, but not LPS (Figure 2.5C). Overall, these data establish that distinct targets of the promiscuous TIRAP lipid-binding domain are functional, and allow this adaptor to promote TLR signaling from more than one site in the cell.

2.4 Discussion

It has become clear in recent years that TLRs can induce signal transduction from diverse locations in the cell, with the cell surface and endosomal membranes being the best-defined sites [79]. However, the question remained as to how this diversification of receptor locale can be accommodated with the common need to activate MyD88-dependent signaling. Our finding that TIRAP can function as a sorting adaptor for MyD88 at the cell surface and endosomes provides a molecular explanation to this question and fills an important gap in our knowledge of how diverse subcellular compartments can support TLR signaling.

Several lines of evidence indicate that TIRAP can function from endosomes. First, TIRAP KO primary and immortal BMDM are defective for TLR signaling in response to HSV infection. This finding is in contrast to results obtained with non-degradable nucleic

acids containing phosphorothioate linkages, which bypass the need for TIRAP in primary BMDM. These data highlight the utility of natural activators of innate immunity to dissect TLR signal transduction pathways. Second, TIRAP is expressed in cells that do not express plasma membrane-localized TLRs (pDCs), and TIRAP KO pDCs are defective for IFN responses induced by TLR7 or TLR9. Because plasma membrane-localized TLRs are not functional in pDCs, these data eliminate any possibility that contaminating TLR2 or TLR4 ligands can explain our findings. Third, TIRAP is a component of myddosomes induced by TLR4 and TLR9, and is required for their formation. Collectively, using multiple activators, assays and cell types, these data establish that TIRAP has a widespread role in controlling TLR signal transduction in response to bacteria and viruses.

We do note however, that not all MyD88-dependent responses are mediated by TIRAP. In addition to the MyD88-dependent signaling pathways activated by the IL-1 receptor (IL-1R) family [152,196], we found that TLR7/9-induced IL-12p40 expression in pDCs was TIRAP-independent. This finding is in contrast to TLR7/9-induced IFNα expression in the same cells. Thus, like TLR4 [169], at least some endosomal TLRs may engage distinct sorting adaptors to induce compartment-specific cellular responses. The sorting adaptor(s) that mediates MyD88 recruitment to these TIRAP-independent receptors remains unknown. An alternative explanation to these observations is that endosomes which induce IL-12p40 expression may contain higher concentrations of TLR ligands than IFN-inducing endosomes, rendering some signaling pathways more dependent on TIRAP than others. While it is formally possible that some TLRs simply recruit MyD88 to their cytosolic TIR domains directly (bypassing the need for a sorting adaptor), the

increasing incidence of sorting adaptor dependence for MyD88 recruitment makes this possibility unlikely.

TIRAP is one of numerous phosphoinositide-binding proteins that can bind to multiple lipids [205]. This promiscuity of lipid binding has been a point of inquiry, as we and others have suggested that PI(4,5)P2 is mainly responsible for controlling its localization and function [155,204]. Our finding that a PI(4,5)P2-specific allele of TIRAP is sufficient to support TLR4 signaling is consistent with this idea. However, PI(4,5)P2-specific alleles of TIRAP cannot support TLR9 signaling. These observations argue strongly that the role of TIRAP in TLR signaling from endosomes involves interactions with lipids other than PI(4,5)P2. Our functional analysis of TIRAP alleles that bind to single lipids revealed that PI(3)P and PS are sufficient to support TLR9 signaling. From these data, it becomes clear that multiple targets of the lipid-binding domain of TIRAP are functionally important.

The analysis of TIRAP alleles that bind single lipids was also informative in considering the precise subcellular sites of TLR signaling. We found that PI(4,5)P2-specific and PS-specific alleles of TIRAP are localized to the plasma membrane, but only PI(4,5)P2 specificity confers LPS responsiveness. Because the PS-specific TIRAP allele is functional when the correct stimulus is applied to the cells (CpG DNA), these data cannot be explained by this protein being misfolded or poorly expressed. Rather, these findings may highlight the precise requirement for TIRAP to be present in a region of the cell surface that is devoid of PS (or at least enriched for PI(4,5)P2). This suggestion is consistent with prior work demonstrating that localization of TIRAP to the plasma

membrane is not sufficient for TLR4 signaling [204]; TIRAP must be localized to PI(4,5)P2-rich regions specifically. Likewise, our analysis of TLR9 signaling highlights the importance of TIRAP localization to endosomes containing PS, the 3' phosphoinositide PI(3)P, and probably PI(3,5)P2 [78]. These observations are of note when considering that the various membrane-bound organelles in mammalian cells have been proposed to consist of a phosphoinositide code (PI code) [209]. This PI code is "read" by various lipid-binding proteins to assemble different protein structures on different membranes, effectively allowing the PI code to determine (in part) the activities of each organelle [209]. Our analysis of this PI code, through the use of TIRAP alleles that bind to single lipids, provides a functional means to identify the precise subcellular sites of TLR signal transduction. We suggest that a comprehensive map of the sites of innate immune signal transduction can be created by further study of the link between the PI code and sorting adaptor localization.

From a broader perspective, this new function for TIRAP in controlling TLR signaling from multiple organelles is reminiscent of the activities of the protein MAVS, the receptor-proximal adaptor in the antiviral RIG-I like Receptor signaling pathway[105]. Both MAVS and TIRAP can be considered sorting adaptors, in that they define the subcellular sites of signal transduction induced by their respective upstream receptors. Like TIRAP, MAVS is located on several organelles in the cell, all of which are important for antiviral signal transduction [62,105,210]. Thus, a common theme appears to be emerging whereby the sites of innate immune signal transduction can be diversified simply by altering the subcellular sites of sorting adaptor residence.

In summary, we have revealed a novel means by which a promiscuous lipid-binding protein can be used in nature. We suggest that the sorting adaptor TIRAP evolved specifically to survey multiple organelles for the presence of activated TLRs. In this context, the promiscuity of lipid binding by TIRAP provides a molecular explanation for how the subcellular sites of TLR signal transduction are so diverse. Our work also demonstrates a novel means by which functional diversity can be achieved at the level of a single protein, as TIRAP can now be considered to function in bacterial detection at the plasma membrane and virus detection in endosomes. These studies therefore highlight how promiscuity, rather than specificity, can be beneficial in biological systems, and provide a mandate to examine the function of other promiscuous lipid-binding proteins in the innate immune system, and beyond.

2.5 Materials and Methods

Cell culture, stable transductions, microscopy

WT (C57B/6) and TIRAP KO iBMDM were a gift from D. Golenbock (UMass) and were cultured in complete DMEM (Gibco) containing 10% FBS and 5% L929 conditioned supernatant. Primary BMDM from WT (C57B/6) or TIRAP KO mice (Jax 017629) were prepared as described [204]. Cells were stimulated LPS (Invivogen) at 100ng/mL or phosphorothioate-linked CpG DNA (TCCATGACGTTCCTGACGTT (MGW Operon) at 1μM, unless otherwise indicated.

TIRAP alleles were introduced into TIRAP KO iBMDM by retroviral transduction and sorted by FACS to normalize GFP expression. When biotin-based myddosome isolations were performed, TIRAP alleles were introduced in iBMDM stably expressing

the biotin ligase BirA. Where indicated, cells were fixed with 2% paraformaldehyde and stained with anti-GFP (Clontech) according to manufacturer's instructions. Antibody staining was detected with the secondary antibody labeled with Alexafluor-488 and analyzed by confocal microscopy.

Primary BMDM were transfected by nucleofection using mouse macrophage transfection reagent (Lonza VPA-1009) according to manufacturer's instructions. Cells were imaged 4 hours later by confocal microscopy. Where indicated, dextran-Alexa Fluor 647 (Life Technologies) was used at 10µg/mL.

Flt3 ligand-induced bone marrow derived pDC culture

Bone marrow cells were isolated and cultured for 7 days in RPMI supplemented with 10% FBS, 50 units/mL penicillin, 50µg/mL streptomycin, 10mM HEPES (Invitrogen) and 2-mercaptoethanol (Sigma) and 100ng/mL Flt3-ligand (GEMINI). On day 8, cells in the supernatant were harvested, counted and plated for subsequent experiments.

Viral infections

KOS A, KOS CE, and KOS K (Sato et al., 2006) viruses were propagated and titered on Vero cells as described [211]. The ICP0-null (7134) and restored (7134R) virus was grown and titred on U2OS cells [212]. Virus was diluted in PBS containing 0.1% (wt/vol) glucose and 1% (vol/vol) heat-inactivated FBS. Cells were infected at the MOI indicated for 1 h at 37 °C, washed twice with PBS, and overlaid with DMEM containing 1% (vol/vol) heat-inactivated FBS. Infected cells were incubated at 37 °C for the indicated length of time. $2x10^6$/well pDCs were stimulated with either $2x10^6$ PFU (plaque forming unit) of HSV-2

(186 syn+) or $2x10^7$ PFU of Influenza A/PR/8 for 24 hours. Supernatant was then harvested and ELISA was performed to measure IFN-α and IL-12p40. BMDM were infected with the Type 3 Dearing strain of reovirus at MOI: 10, 100 or 1000 for 3 hours, and analyzed by qPCR as described below.

Microarray sample generation, data acquisition and processing

pDC and cDC samples for microarrays were generated as described [213]. In brief, DPE-GFPxRAG1$^{-/-}$ transgenic mice [214,215], in which pDCs are identified by GFP expression (Iparraguirre et al., 2008) were injected with Flt-3ligand-expressing B16F10 tumor cells for expansion of DC subsets. 12-14 days later, spleens were harvested and stained for CD11b and CD11c. pDCs and cDCs were sorted using a MoFlo cell sorter (DakoCytomation) based on high GFP expression and a CD11c^{hi}CD11b^{hi} phenotype, respectively. Samples were prepared in 2 independent experiments. Total RNA, isolated with the RNeasy kit (Qiagen), was processed, amplified, labeled and hybridized to mouse MOE430v2 GeneChip microarrays (Affymetrix) by the University of Pennsylvania Microarray Core Facility using standard protocols. Data were processed using Affymetrix GeneChip Operating System v1.4 software, and probe intensity files (.cel files) were imported and analyzed in Partek Genomics Suite (v6.6, Partek). Data were $\log_2$-transformed and normalized with GC Robust Multi-Array Averaging (GCRMA). Significance Analysis of Microarrays (SAM, samr v2.0, Stanford University) [216] was applied to identify differentially expressed genes in mDCs and pDCs. The most significant probe set for each gene of interest was used to calculate fold-changes (in Partek) between gene expression levels in mDCs versus pDCs that were graphed using

Prism software (v. 5.0c, GraphPad Software). Microarray data sets are available through the Gene Expression Omnibus (GEO); accession number GSE50436.

Phagocytosis assay

$5x10^5$ cells were kept on ice in 500uL of complete media (described above) and incubated with Fluoresbrite Carboxy YG 2.0 micron beads (PolySciences) at 10 beads/cell. Cells were added to a 37^oC water bath for indicated times. Phagocytosis was stopped with ice-cold PBS. Cells were washed 3x with ice-cold PBS, and analyzed by FACS.

Myddosome isolation assay

Cells were plated on 10cm tissue-culture treated dishes and grown to confluency (10^7 cells/plate overnight). Cells were stimulated with ligand as indicated, then lysed in 700µL of buffer containing 1% NP-40, 50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 10% glycerol and protease/phosphatase inhibitors (Roche). 100 µL of cleared lysate was retained for analysis (input) and remaining 600µL was incubated overnight at 4^oC with 1µg anti-MyD88 (R&D sciences) or anti-IRAK2 (ProSci). The following day, 50µL of protein G sepharose (GE healthcare) was added for 1 hour. Alternatively, cleared lysates were incubated with Neutravidin agarose beads (Thermo) for 2 hours. Beads were washed 3x with lysis buffer, then proteins were extracted by adding 50µL 2x Laemmli buffer, electrophoresed and immunoblotted with the indicated antibodies using standard conditions. The following antibodies were used: anti-MyD88 (R&D), anti-IRAK2 (Prosci), anti-HA (3F10, Roche), anti-GFP (JL-8, Clontech) and anti-actin (ac-15, Sigma). Anti-IRAK4 was kindly provided by Shizuo Akira.

Real time quantitative PCR

Total RNA was extracted from 2×10^6 cells using RNA Bee (Tel-Test Inc) according to manufacturer's instructions and analyzed with TaqMan one-step qPCR reagents. Expression was plotted relative to GAPDH, shown as mean and standard deviation of 3 technical replicates. Each graph is representative of at least 3 independent experiments.

Plasmids

TIRAP-TAP IRES GFP was produced as follows. Human TIRAP was appended with 3xHA and a BirA target site [217,218] by overlap extension PCR. The biotin ligase BirA [218] was subcloned into pMSCV2.2 by standard procedures. WT TIRAP-GFP, PLC-TIRAP-GFP and PX-TIRAP-GFP were described previously [204], and are present in the retroviral vector MSCV202 IRES-hCD2. pEGFP-N1-based TIRAP loc-GFP (amino acids 1-85 of TIRAP fused in frame to GFP) was described [204]. SLP2a-TIRAP-GFP was cloned similarly to the TIRAP alleles described above. Briefly, the C2A domain of murine SLP2a was cloned from a murine spleen cDNA library and fused in frame to amino acid 86 of TIRAP-GFP. This SLP2a-TIRAP-GFP fusion cDNA was then subcloned into the retroviral vector MSCV2.2 IRES-hCD2. mCherry-PH was purchased from Addgene (plasmid 36075).

Chapter 3: Loss of TIRAP Links Endotoxin Tolerance and Superinfection

3.1 Abstract

The inflammatory response evolved to clear our bodies of perceived threats such as bacteria or viruses. This inflammatory response can also result in “innocent bystander” effects where our own tissues can become damaged. To prevent tissue damage and other injury, the immune system has evolved negative feedback mechanisms to prevent runaway activation and limit the deleterious effects of inflammation. However, one major consequence of negative regulation of inflammation is a decreased ability to respond to repeated infections. Here, we show a novel regulatory mechanism by which multiple infectious stimuli cause cells to degrade TIRAP, a critical adaptor downstream of TLRs, resulting in suppression of TLR responses that normally lead to inflammation. Degradation of TIRAP requires two independent stimuli: a TLR signaling event and exposure to type-1 IFNs. We show that TLR4, which is known to lead to both of these signals, results in degradation of TIRAP and an “endotoxin-tolerant” state. However, signaling through other TLRs in response to viral infection, which also stimulates cells to produce IFNs, can serve as the necessary triggers for TIRAP degradation and negative regulation of TLR signaling pathways. The data presented in this chapter describe a previously unrecognized regulatory mechanism for altering the inflammatory response after initial TLR stimulation and may have implications for treatment of sepsis and microbial super infection.

3.2 Introduction

Though inflammation plays a critical role in the normal clearance of infectious microorganisms, it can also cause severe damage to host tissues. Numerous examples of pathophysiology have been linked to excess inflammation including sepsis, a constellation of clinical symptoms associated with disseminated bacterial infections [219]. Despite decades of history in treating septic patients, few clinical interventions are successful, and sepsis remains a leading cause of death and one of the leading monetary expenditures in hospitals around the world.

Many negative regulatory mechanisms have evolved to reign in the damage caused by excessive inflammation, including endotoxin tolerance (ET) [220]. First described more than 60 years ago, ET describes a modulation of responsiveness to the bacterial outer membrane molecule lipopolysaccharide (LPS). Innate immune cells detect LPS through toll-like receptor 4 (TLR4), and when exposed to small amounts of LPS, undergo numerous regulatory and transcriptional changes that lead to a decrease in proinflammatory cytokine expression following subsequent stimulation [221]. However, ET cells are not simply unresponsive to LPS. Instead they have an altered transcriptional response characterized by an increase in phagocytic and degradative potential and the production of anti-inflammatory cytokines. Understanding ET could improve therapeutic intervention in sepsis, yet despite decades of study, the molecular basis of endotoxin tolerance is still poorly understood [222].

Still, numerous molecular mechanisms for ET have been proposed. For example, transcription factors activated by TLRs induce the transcription of negative regulators of

TLR signaling such as IRAK-M [223]. Expression of IRAK-M correlates with ET, and genetic ablation at this locus leads to increased inflammatory responses in models of sepsis [224,225]. Though IRAK-M knockout animals are incapable of inducing endotoxin tolerance, and IRAK-M is unique among negative regulators linked with ET in its demonstrated involvement in both mouse and human ET [220,226], further characterization of the mechanistic link between IRAK-M and ET remains lacking. In addition, interpreting the results of the genetic ablation of negative regulators like IRAK-M is complicated due to the tendency of these regulators to act early in the acute response. For example, IRAK-M knockout animals have elevated gene expression of proinflammatory cytokines within hours of LPS encounter [225]. As ET is typically evaluated after 24-48 hours, these genetically altered ET cells have experienced a fundamentally different constellation of responses than WT cells.

As with bacterial sepsis, inflammation induced by acute viral infections can be deadly, but many viral infections also predispose their hosts to subsequent bacterial infections. For example, influenza kills between 5,000 and 50,000 people in the United States each year, but the majority of mortality is associated with secondary pneumococcal infections [227]. The threat of these so-called "superinfections" raises a regulatory paradox for the immune system, since inhibiting inflammation to block damage caused by viral infections may render and individual susceptible to new infectious assault. Understanding the balance between inflammation and superinfection also raises a paradox for investigators, and despite its clinical importance, molecular understanding of the phenomenon of superinfection is also lacking [227,228]. Many pathogens target innate immune responses for inhibition directly, making it difficult to distinguish host

regulatory responses from viral virulence strategies [109,130]. In addition, responses to infections *in vivo* are complicated and varied, and alterations to innate responses may alter pathogen load and affect the outcome of infection for reasons unrelated to the pathways being investigated. Further, restoring the ability of cells to respond to secondary infection may lead to increased inflammatory tissue damage that will have adverse effects in the context of the primary infection.

Still, as with ET, several potential mechanisms have been proposed. In the case of influenza, the most commonly accepted explanation is that damage to epithelial barriers increases potential sites of attachment for pathogenic microbes [229,230]. Yet seasonal human influenza does not cause severe lung injury [227], and mouse models demonstrate that susceptibility to superinfection can persist even after viral clearance[228]. Other explanations include decreased monocyte chemotaxis [231], as well as neutrophil and natural killer (NK) cell dysfunction [228,232]. Unfortunately, these studies are primarily descriptive. For example, in the study implicating NK cell dysfunction, adoptive transfer of naïve NK cells could protect from subsequent bacterial infection, but no explanation for the dysfunction of influenza-experienced cells was provided.

Here, we report a previously uncharacterized link between the phenomena of ET and superinfection. By investigating the earliest events in TLR signaling in macrophages, we show that endotoxin tolerant macrophages lose expression of TIRAP, a key component of the signaling pathway downstream of most toll-like receptors. This down regulation requires two signals, MyD88-dependent NFκB activation, and type-1 IFN signaling. Both of these signals may be provided by activation of TLR4, which initiates two parallel

signaling pathways, the MyD88-dependent pathway activating NFκB, and the TRIF-dependent pathway culminating in the production of type-1 IFN. These signals may also be provided by viral infections, which activate endosomal TLRs to provide the MyD88-dependent response, and activate cytosolic pattern recognition receptors such as RIG-I to generate IFN. Together, these data suggest that TIRAP is a key focal point of negative regulation in toll-like receptor signaling.

3.3 Results and Discussion

TIRAP is lost in endotoxin tolerant macrophages

To determine the molecular basis of endotoxin tolerance in TLR signaling, we investigated the ability of ET macrophages to form a myddosome in response to LPS. This approach has several advantages. First, in contrast to monitoring gene expression, which may be influenced by a myriad of regulatory processes, monitoring formation of the myddosome allows us to investigate the earliest intracellular event in the signaling pathway and thus reduces the number of factors that may influence the results. Second, myddosome assembly can be monitored in WT or stably transduced cells, allowing investigation without substantial perturbations of the cellular response. Finally, the regulation of early signaling events is likely to have the largest effect on downstream responses. Thus, the formation of the myddosome may provide a useful window into negative regulation in ET.

Naïve or ET immortal macrophages were stimulated with LPS, and cells were monitored for their ability to form myddosome complexes. In naïve macrophages, MyD88 rapidly associates with IRAK4 and IRAK2 in response to LPS. By contrast, pretreatment of

macrophages with a low dose of LPS rendered cells unable to form myddosome complexes in response to further LPS treatment (Figure 3.1A). Because LPS stimulation results in many changes in gene expression, it is reasonable to consider that components of the TLR4 pathway may be down regulated in ET. We therefore examined the cell surface expression of TLR4 in naïve and ET macrophages. Naïve macrophages express high levels of TLR4, but rapidly internalize the receptor in response to LPS. ET macrophages displayed similar behavior (Figure 3.1B). This is inconsistent with a report that suggests that TLR4 is downregulated in ET peritoneal macrophages [139]. This may reflect a difference between peritoneal and bone marrow-derrived macrophages, or the behavior of the antibody used. The epitope recognized by clone MTS510 (used by Nomura *et. al.*) is masked when the receptor is bound to LPS, while that used in this study (SA15-21) is not [159,233]. It is also worth noting that ET macrophages have altered, but not absent responses to LPS [234], suggesting that they are still capable of detecting LPS through TLR4. Taken together, these data suggest that decreased TLR4 is not a likely explanation for ET. We therefore examined the expression of the constituents of the myddosome in ET versus naïve macrophages. Apparent concentrations of MyD88 remained constant over 16 hours of LPS stimulation (Figure 3.1C) However, we observed that ET immortalized macrophages exhibit significantly reduced TIRAP protein compared with naïve cells, beginning approximately 12 hours after initial LPS treatment.

As TIRAP is a critical component for signaling downstream of TLR4 from the cell surface, but not within endosomes [154,235], decreased TIRAP expression provides a plausible mechanism for development of ET following LPS stimulation, since TRIF-

Figure 3.1

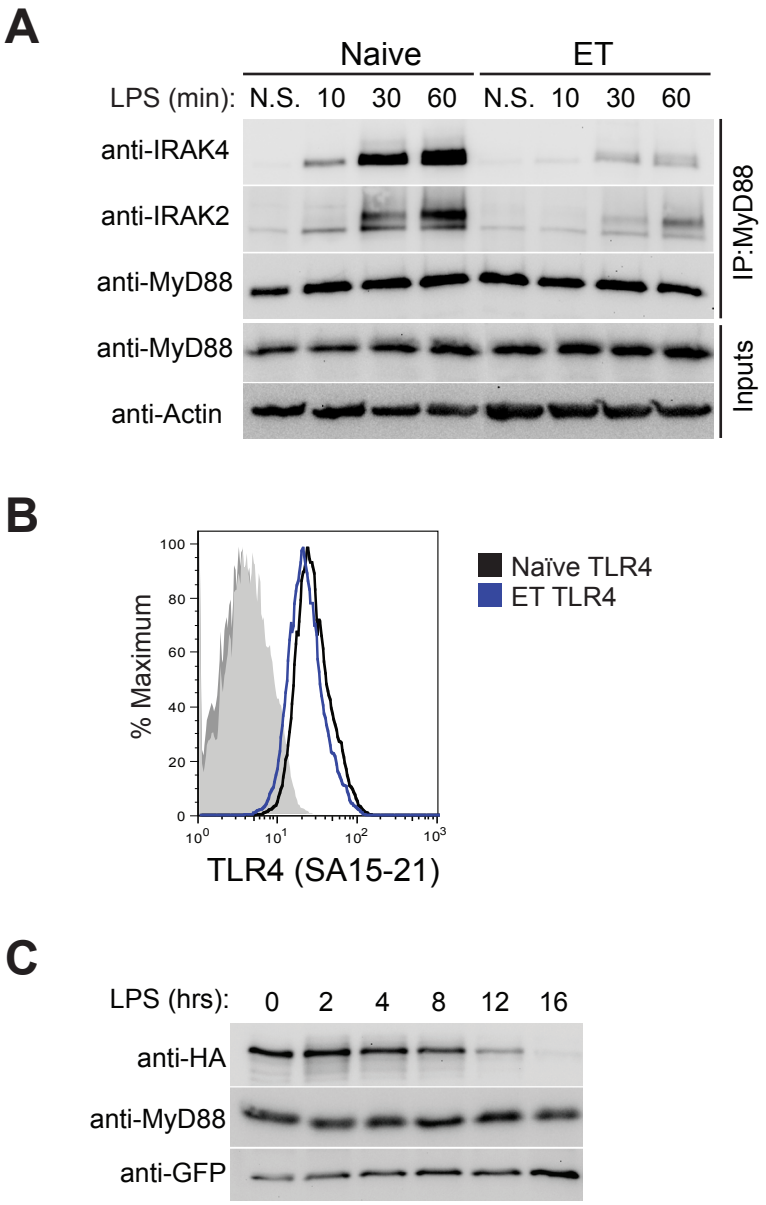

Figure 3.1 Endotoxin Tolerant Macrohages are Defective for Myddosome Formation
(A) WT and ET macrophages were stiumlated with LPS and assed for the ability for form myddosome complexes by immunoprecipitation. ET Macrophages are significantly impaired in their ability to recruit IRAK4 and IRAK2 to MyD88.
(B) WT and ET macrophages were stained for surface TLR4 expression (SA15-21) compared to unstained controls (grey histograms). WT and ET macrophages have equivalent levels of TLR4 surface expression.
(C) HA-TIRAP exressing macrophages were stimulated with 1ng/mL LPS and lysates were acquired at different times. TIRAP expression is noticably decreased by 12 hours and nearly undetectable by 16 hours.

dependent signaling should be unaffected. Further, while many negative regulators implicated in ET are expressed within hours of LPS treatment, reduction of TIRAP concentration is not observed until approximately 12 hours post stimulation (Figure 3.1C). This suggests that this form of regulation is unique to ET, and does not participate in the acute response.

Loss of TIRAP is Likely Due to Degradation

The reduction of apparent TIRAP concentration in ET could be explained by reduced transcription, post-transcriptional regulation such as that mediated by miRNAs or degradation of the translated protein. However, transcript levels were unchanged in ET macrophages compared to their naïve counterparts (Figure 3.2A). Further, since TIRAP is ectopically expressed under a constitutive promoter in the cells examined [65], loss of TIRAP is not likely due to changes in transcription. Similarly, translational regulation by miRNAs is also unlikely to explain loss of TIRAP in ET.

According to targetscan.org, the only miRNA predicted to interact with TIRAP mRNA in humans and mice is miR-142-3p [236]. Though this miRNA has been implicated in TLR responses [237], it is expressed at steady-state in dendritic cells (DCs)[238], which suggests that its regulatory function is exerted prior to activation of TLR signaling. Further, miR-142-3p is predicted to interact with the 3' untranslated region (UTR) of the TIRAP mRNA, which is not present on the HA-TIRAP construct used in Figure 3.1C. Targeting the 3'UTR is also typical for miRNAs generally [239]. Though this does not rule out the possibility that TIRAP is subjected to miRNA regulation, these observations suggest that miRNAs are not a likely candidate for explaining loss of TIRAP in ET. In order to truly

Figure 3.2

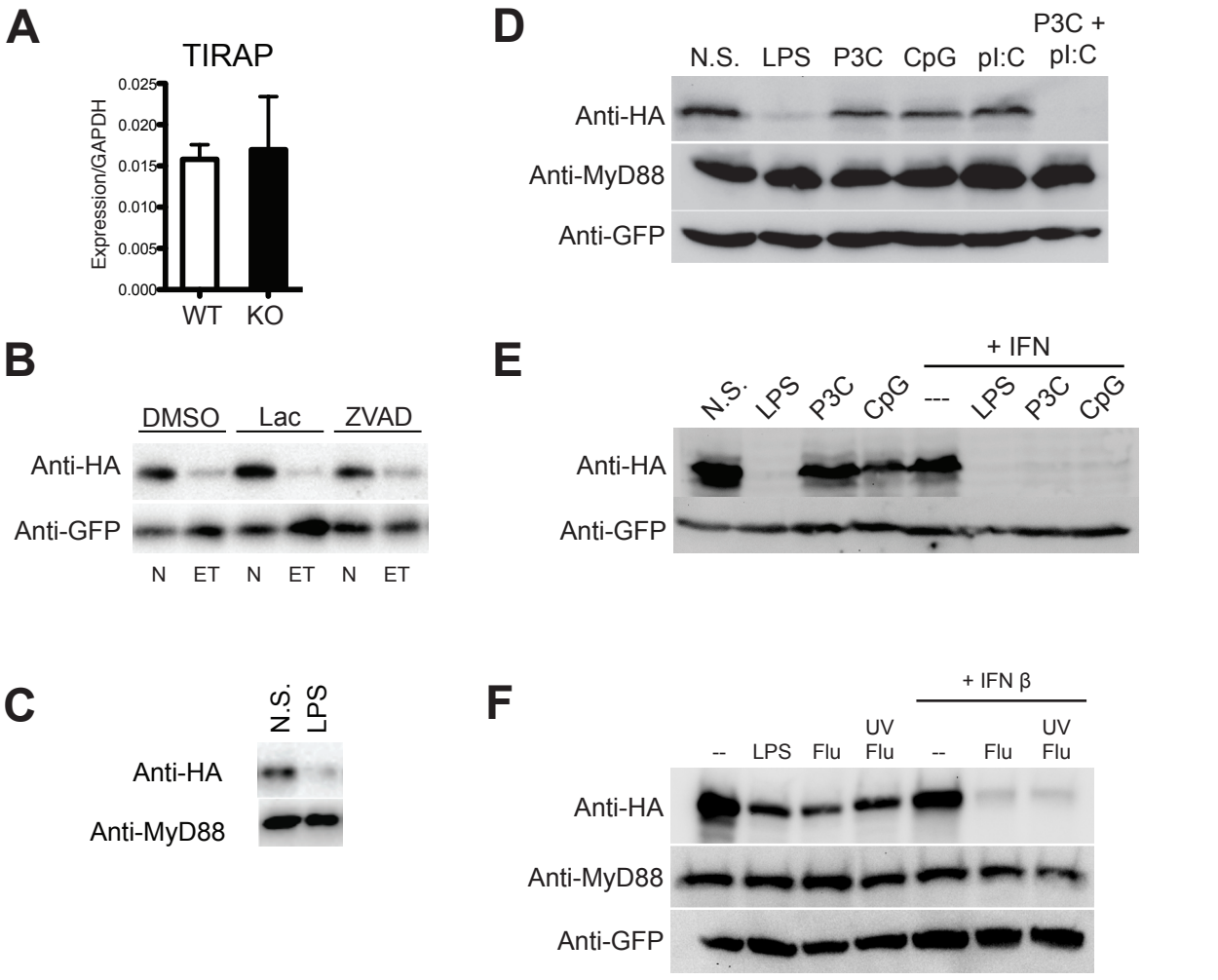

Figure 3.2 TIRAP Loss in ET requires MyD88-dependent signaling and type-1 IFN
(A) Naive and ET macrophages were assessed for TIRAP expression by qPCR
(B) HA-TIRAP expressing macrophages were either treated (ET) or left untreated (N) for a total of 20 hours hours. At 12 hours post-treatment, cells were treated with the proteasome inhibitor lactacystin, the caspase inhibitor ZVAD or vehical (DMSO). The presence of HA-TIRAP was assessed by western blot.
(C) Cells expressing PLC-TIRAP-HA were treated with 10ng/mL LPS for 24 hours and assessed for loss of the HA-expressing construct.
(D-F) HA-TIRAP expressing macrophages were treated with different combinations of TLR ligands (D) as well as recombinant IFNβ (E) or influenza (F) PR8 strain for 24 hours and assessed for the presence of TIRAP. Note that both a TLR signal and a source of type-1 IFN is required for loss of TIRAP.

rule out the role of miRNAs in TIRAP loss, one could monitor loss of TIRAP in DICER knockdown macrophages, which should be defective for all miRNA expression[240].

The final possibility is that TIRAP is targeted for degradation in ET. Indeed, TIRAP is known to be the target of proteasomal degradation and cleavage by caspase-1 [164,165]. In order to determine if proteasomal degradation plays a role in ET-induced TIRAP loss, we treated macrophages with pharmacological inhibitors of these pathways during the induction of ET (Figure 3.2B). Unfortunately, these preliminary experiments were inconclusive. It will be possible to further examine whether TIRAP loss is the result of degradation through mutagenesis studies. An allele of TIRAP in which the n-terminal localization domain is replaced with the lipid binding domain of phospholipase C δ1 (PLCδ1) (see Chapter 2) was lost in ET macrophages (Figure 3.2C), suggesting that any target of degradation must reside in the c-terminal TIR domain. Caspase-1 cleaves TIRAP in the TIR domain at amino acid D198, and a substitution of this residue for alanine renders the protein insensitive to cleavage. In principal, it should be possible to monitor loss of this mutant allele in ET macrophages to determine whether caspase cleavage is important. However, this mutant allele is not capable of rescuing signaling in TIRAP knockout macrophages, and caspase-1 knockout macrophages appear to be defective for TIRAP-dependent responses [165], suggesting that cleavage is somehow critical for TIRAP's function. Since degradation of TIRAP may require participation in a signaling complex, negative results in this proposed experiment will be difficult to interpret. Investigating degradation by the proteasome may be equally problematic. Proteins are targeted for proteasomal degradation by the attachment of polybuquitin chains to lysine residues [241]. The TIR domain of human TIRAP contains 3 lysines [153],

which could be mutated to arginines to abolish polyubiquitin attachment. If this mutant allele is not lost in ET, presuming that it is capable of participating in signaling downstream of TLR4, this result would suggest that proteasomal degradation is responsible.

Loss of TIRAP Requires TLR signaling and type-1 interferon

We next considered what signaling events downstream of LPS might initiate the loss of TIRAP. LPS binding to TLR4 activates two distinct signaling pathways: a MyD88-dependent pathway that activates NFκB and AP-1 transcription factors and the production of pro-inflammatory cytokines, and a TRIF-dependent signaling cascade culminating in the activation of IRF3 and the production of type-1 IFN [149]. Aside from TLR4, all TLRs activate either MyD88-dependent or TRIF-dependent signaling but not both [91]. It is therefore possible to study these pathways in isolation. To determine whether MyD88 signaling alone was sufficient to induce loss of TIRAP, we chose to investigate TLR2 and TLR7, both of which activate the MyD88-dependent pathway. Treatment of macrophages with the TLR2 ligand PAM3CSK or the TLR9 ligand CpG DNA was not sufficient to induce loss of TIRAP (Figure 3.2D). To determine whether TRIF signaling alone is sufficient, macrophages were treated with poly I:C, which activates the TRIF-dependent pathway downstream of TLR3. As with MyD88-dependent signaling, TRIF signaling alone was not sufficient to induce loss of TIRAP. Strikingly, though neither MyD88- nor TRIF-dependent signaling was sufficient, simultaneous induction of both pathways by treatment with poly I:C and CpG DNA caused loss of TIRAP comparable to that caused by LPS treatment (Figure 3.2D).

As noted above, MyD88-dependent signaling principally activates NFκB and AP-1 transcription factors, while TRIF-dependent signaling principally activates IRF3 and type-1 IFN. The requirement for both signaling pathways suggests a number of possibilities. First, the production of the TIRAP eliminating factor(s) may require the activation of multiple transcription factors. However, though TRIF signaling downstream of TLR3 uniquely activates IRF3, it is also capable of activating NFκB and AP-1, suggesting that there is something unique about MyD88-dependent signaling other than its activation of transcription factors. Since TIRAP is involved in all MyD88 dependent signaling that have been described [65], this may imply that TIRAP must be involved in signaling in order to be targeted. One way to test this would be to express a mutant allele of TIRAP that cannot interact with the TIR domain of TLR4, which should resist elimination in ET if this hypothesis is correct. Another possibility is that some product of TRIF signaling, rather than activation of IRF3 is sufficient. Since type-1 interferon is the principal secreted factor downstream of IRF3, and because IFN receptor (IFNAR) knockout mouse macrophages are resistant to tolerance induction [242], we hypothesized that IFN alone might substitute for TRIF-dependent signaling. Indeed, treatment of macrophages with recombinant IFNβ could substitute for TLR3-mediated TRIF signaling and induce loss of TIRAP in combination with either TLR2 or TLR9 signaling (Figure 3.2E), suggesting that TRIF signaling itself is dispensable in ET provided cells are in the presence of type-1 IFN.

Since MyD88-dependent signaling in the presence of IFN is not unique to LPS signaling, we next considered whether TIRAP loss could be observed in response to other stimuli. In particular, many viral infections trigger MyD88-dependent TLR signaling in

macrophages, as well as the production of type-1 IFN downstream of cytosolic RNA or DNA sensors [94,243]. This possibility is especially intriguing since viral infections such as influenza may render their hosts more susceptible to bacterial infection [227], a phenomenon that could be partially explained by loss of TIRAP and insensitivity to TLR ligands. In addition, influenza infection desensitizes mice to TLR stimulation, though an unknown mechanism [244]. To assess whether viral infections could induce loss of TIRAP, HA-TIRAP expressing immortalized macrophages were infected with the mouse-adapted influenza strain PR8 and assessed for the presence of TIRAP. As expected, PR8-infected macrophages lost TIRAP to a similar extent as ET macrophages (Figure 3.2F). This loss was further exacerbated by the addition of exogenous recombinant IFNβ.

TIRAP Modulates Responses to Influenza In Vivo

The role of TLR signaling in the clearance of influenza is unclear. In one study, either MyD88- or MAVS-dependent signaling alone was sufficient to control infection, while mice doubly deficient for both MAVS and MyD88 were susceptible[243]. Contradicting this finding, a similar study showed that TLR7 or MyD88 KO mice were significantly more susceptible to influenza than their WT counterparts[245]. Yet another study demonstrated that TLR7 knockout mice were no more susceptible to influenza infection, but were moderately protected from secondary bacterial infection after influenza challenge [246]. This latter result supports our hypothesis that TIRAP loss may be important in superinfection, since TLR7 KO mice should be unable to provide the MyD88-dependent signal required to cause loss of TIRAP.

To date, no one has assessed the role of TIRAP in influenza infection. In order to assess the role of TIRAP in controlling influenza infection *in* vivo, WT and TIRAP knockout animals were infected intratracheally at LD80 with strain A/Puerto Rico/8/1934 H1N1 (PR8), and assessed daily for weight loss[247]. 80% initial body weight was the clinical endpoint. WT animals dropped to 80% initial bodyweight by day 8 post infection (n=6), while TIRAP knockout animals exhibited a slight decrease in body weight but began to recover after 8 days (n=5) (Figures 3.3A). In accordance with the protocol, all WT mice were sacrificed on day 8 at 80% initial body weight, while TIRAP KO mice appeared to recover (Figure 3.3B). These results suggest that the inflammation induced by TLR responses to influenza may contribute to viral damage, and loss of TIRAP could be a regulatory strategy to reduce this damage.

This hypothesis raises several questions that have yet to be tested. Are TIRAP KO mice still able to induce robust adaptive immune responses? The role of TLR signaling in the production of protective antibodies is contested. One study showed that loss of MyD88 or TLR7 altered antibody isotype skewing, but had no effect on protection from secondary challenge, while a similar study showed that the same knockout mice were significantly more susceptible to secondary challenge [243,248]. Does TLR signaling help control viral load? As noted above, MyD88 and TLR7 deficient mice are similarly able to control primary challenge, provided RLR signaling is intact. However, all of these studies use different initial viral load and route of administration, so direct comparison is problematic.

Figure 3.3

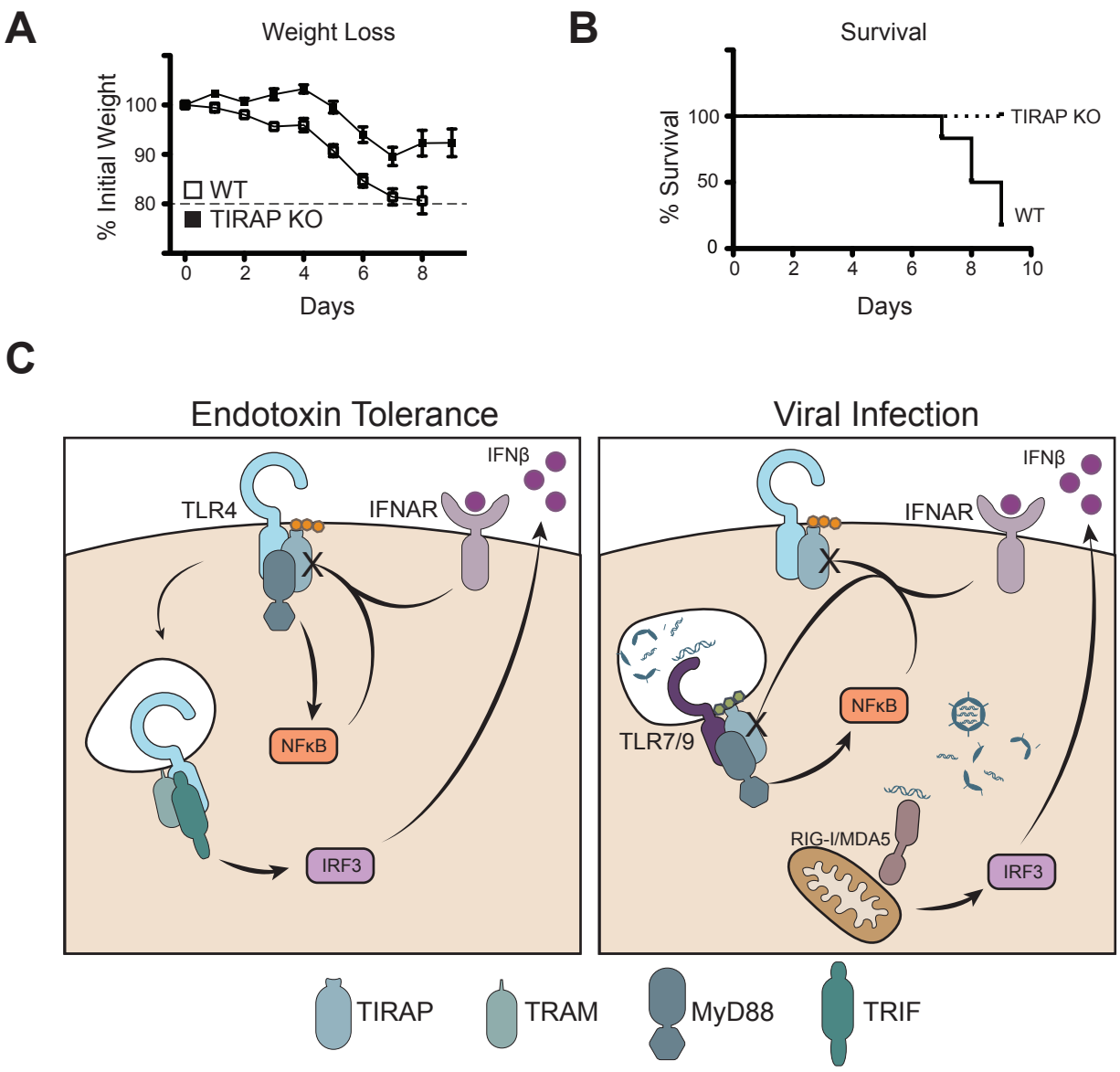

Figure 3.3 TIRAP Modulates Responses to Influenza Infection
(A-B) WT or TIRAP KO mice were infected with influenza (LD 80) and monitored for weight loss. Mice were sacrificed at 80% initial body weight.
(C) Model of signals linking endotoxin tolerance to superinfection via degredation of TIRAP. Endotoxin stimulation (left panel) leads to activation of NFκB through MyD88 and activation of IRF3 through TRIF. Type 1 IFN produced by IRF3 provides the second signal to target TIRAP for degradation. During viral infection (right panel), IFN is provided through activation of IRFs by cytosolic nucleic acid sensors or by other cells such as pDCs (not depicted).

Taken together, these data suggest a possible link between ET and virus-induced superinfection, since viral infection provides the signals required for ET-mediated TIRAP loss (Figure 3.3C). Once the mechanism of TIRAP degradation is identified, this link could be validated by expression of an allele of TIRAP that resists degradation in ET, or removal of a factor responsible for this degradation. We would predict that blocking TIRAP loss in ET would protect against superinfection, though this might increase inflammation-related pathologies of the viral infection itself. These results are particularly exciting in light of the clinical challenges of superinfection. Though the increase in susceptibility to bacterial infection is likely multifactorial, regulation of TIRAP may be a promising target of pharmacologic inhibition. Enhancing immune responses is often problematic, since lowering the threshold of inflammation can lead to inflammatory pathology. However, because TIRAP loss does not appear to regulate acute inflammatory responses, disruption of this regulatory pathway is less likely to promote spurious responses.

3.4 Materials and Methods

Cell culture, stable transductions

WT (C57B/6) and TIRAP KO iBMDM were a gift from D. Golenbock (UMass) and were cultured in complete DMEM (Gibco) containing 10% FBS and 5% L929 conditioned supernatant. Primary BMDM from WT (C57B/6) or TIRAP KO mice (Jax 017629) were prepared as described (Kagan and Medzhitov, 2006). Cells were stimulated LPS (Invivogen) at 100ng/mL or phosphorothioate-linked CpG DNA (TCCATGACGTTCCTGACGTT (MGW Operon) at 1μM, unless otherwise indicated.

TIRAP alleles were introduced into TIRAP KO iBMDM by retroviral transduction and sorted by FACS to normalize GFP expression. Unless otherwise stated, endotoxin tolerant macrophages were generated by culturing cells with 10ng/mL for 24 hours.

Myddosome isolation assay

Cells were plated on 10cm tissue-culture treated dishes and grown to confluency (10^7 cells/plate overnight). Cells were stimulated with ligand as indicated, then lysed in 700µL of buffer containing 1% NP-40, 50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 10% glycerol and protease/phosphatase inhibitors (Roche). 100 µL of cleared lysate was retained for analysis (input) and remaining 600µL was incubated overnight at 4^{o}C with 1µg anti-MyD88 (R&D sciences) or anti-IRAK2 (ProSci). The following day, 50µL of protein G sepharose (GE healthcare) was added for 1 hour. Alternatively, cleared lysates were incubated with Neutravidin agarose beads (Thermo) for 2 hours. Beads were washed 3x with lysis buffer, then proteins were extracted by adding 50µL 2x Laemmli buffer, electrophoresed and immunoblotted with the indicated antibodies using standard conditions. The following antibodies were used: anti-MyD88 (R&D), anti-IRAK2 (Prosci), anti-HA (3F10, Roche), anti-GFP (JL-8, Clontech) and anti-actin (ac-15, Sigma). Anti-IRAK4 was kindly provided by Shizuo Akira.

TLR4 Surface Expression

WT macrophages were treated with 10ng/mL LPS or left untreated for 24 hours. Cells were washed and incubated with DMEM for 30 minutes, and then stained with anti-TLR4 (SA15-21) PE [159,233].

Real time quantitative PCR

Total RNA was extracted from 2×10^6 cells using RNA Bee (Tel-Test Inc) according to manufacturer's instructions and analyzed with TaqMan one-step qPCR reagents. Expression was plotted relative to GAPDH, shown as mean and standard deviation of 3 technical replicates. Graph is representative of 2 independent experiments.

Plasmids

TIRAP-TAP IRES GFP was produced as follows. Human TIRAP was appended with 3xHA and a BirA target site (de Boer et al., 2003; Mechold et al., 2005) by overlap extension PCR. The biotin ligase BirA (Mechold et al., 2005) was subcloned into pMSCV2.2 by standard procedures.

Influenza infections

WT (C57/B6) and TIRAP KO (Jax 017629) were infected intratracheally at LD80 with strain A/Puerto Rico/8/1934 H1N1 (PR8), and assessed daily for weight loss. 80% initial body weight was the clinical endpoint [247]. Macrophages were infected at a multiplicity of infection of 100.

Chapter 4: Perspectives on the Future of Innate Immune Signaling Research

4.1 Introduction

Twenty-five years ago, Charles Janeway stood in front of a room full of immunologists, flatly declared that there would be no further revolutionary changes in our understanding of immune recognition and then immediately proceeded to launch just such a revolution. Our understanding of microbial recognition by the germline encoded pattern recognition receptors that Janeway predicted has changed how we perceive adaptive immune responses, self versus non-self recognition, immunological development and much more. Many of the advances in innate immunity research are the product of the modern genomics era, where gene knockouts are commonplace, and computational approaches can identify structurally related factors. However, as I stated in the introduction to this document, I believe we are approaching another asymptote, where genetic dissection of innate immune signaling pathways will no longer yield straightforward results.

Yet many exciting research avenues remain, where understanding will lead to profound changes in human health. In particular, we must move away from studying individual genes or gene products in isolation, and move towards understanding the complex interactions in their natural context. For example, understanding how compartmentalization within an individual cell governs the behavior of signaling pathways may yield insight into how the quality and magnitude of responses to particular ligands in particular contexts is determined (Chapter 4.2). These same studies may also improve our understanding of cell-type specific responses, since certain cell

types may contain the same basic protein components, while organizing them differently within the cell for different responses. Another fruitful investigative path is in understanding the mechanisms for integrating multiple signals to coordinate a unified response to pathogens. Our innate immune cells did not evolve to respond to purified LPS or synthetic nucleic acid oligos, but must instead interrogate many PAMPs from the same pathogen and use this complex mix of signals to provide the appropriate response (Chapter 4.3). This response may be complicated by the presence of multiple pathogens, as in the case of superinfection, as well as the presence of non-infectious microbes that nevertheless present PAMPs. Finally, the insight gained from basic studies of the innate immune system must be translated to improve human health. Despite 25 years of study into the mechanisms of innate immune detection, no new vaccine adjuvants have been approved for use in the United States, and few therapies incorporate an understanding of the earliest events in an immune response. In addition, the etiology of inflammatory disorders and autoimmune disorders remains unclear, though they must involve an initial inflammatory insult mediated by the innate immune system.

4.2 Cellular Compartmentalization and Innate Immune Signaling

Localization of TLR Sorting Adaptors

In Chapter 2, we demonstrated that the compartmentalization of TIRAP is more complicated than previously appreciated (Figure 4.1A). Though TIRAP can bind to multiple lipids *in* vitro, its lipid binding domain was initially thought to bind exclusively to PI(4,5)P2 *in vivo*, localizing it to the plasma membrane [204]. There were several reasons

Figure 4.1

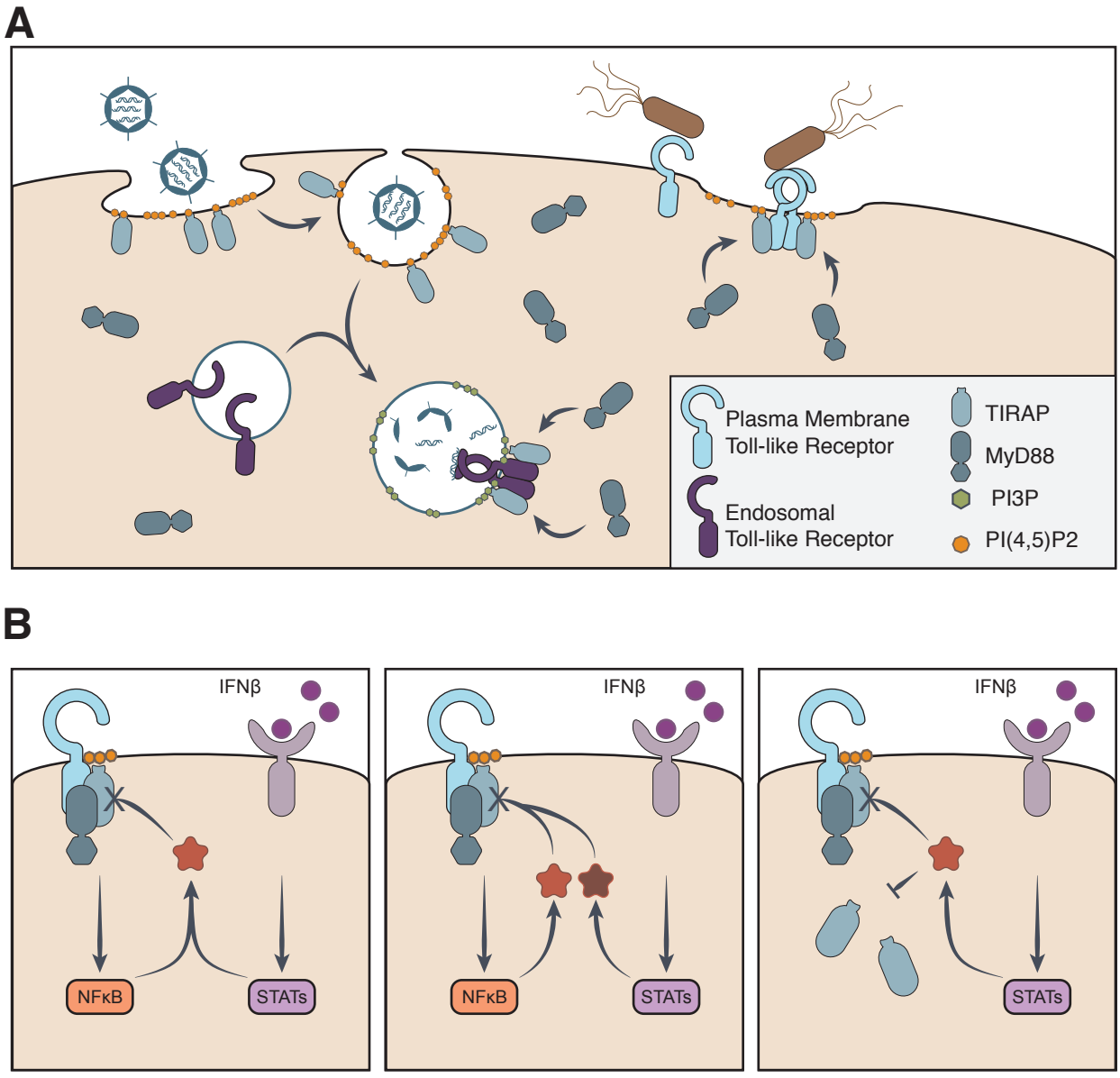

Figure 4.1
(A) Model for the role of TIRAP in TLR signaling from the plasma membrane and endosomes. TIRAP is capable of binding to multiple lipid species to facillitate recruitment of MyD88 to multiple subcellular locations
(B) Three hypothetical models for the regulation of TIRAP degradation in endotoxin tolerance and super-infection. Transcription factors downstream of both TLRs and IFNAR may be required to produce a single factor that targets TIRAP for degradation (left panel). Alternatively, each signaling pathway may produce factors that are individually required to target TIRAP (center panel). Finally, IFN signaling may produce a factor that targets TIRAP, but only when it is in an active signaling complex (right panel)

for the original interpretation. First, though many phophoinositide-binding proteins have promiscuous binding *in vitro*, many have higher affinity for a single lipid species, which determines their *in vivo* function [205]. Second, an allele of TIRAP in which the lipid binding domain was replaced with that of PLCδ1, which exclusively binds PI(4,5)P2, was capable of rescuing all known functions of TIRAP. However, the novel finding that TIRAP is required for signaling downstream of endosomal TLRs called this interpretation into question (Chapter 2 and [65]). Since PI(4,5)P2 is primarily found at the plasma membrane, PI(4,5)P2-localized TIRAP should not be able to function from endosomal compartments. Indeed, we found that PLC-TIRAP was not capable of rescuing signaling downstream of the endosomal TLR9, while an allele of TIRAP that localized to PI(3)P (primarily localized to endosomes) or PS could signal downstream of TLR9, but not TLR4 (Figure 2.5).

TIRAP binds to PI(3)P and PS *in vitro* (Figure 2.4), but the ability of multiple lipid-binding domains to rescue the endosomal signaling of TIRAP does not prove that these are the natural lipid targets *in vivo*, as it is possible that any endosomal localization is sufficient for TIRAP's ability to signal downstream from this location. If true, this would seem to be in contrast to TIRAP's plasma membrane function, since targeting TIRAP to the plasma membrane is not sufficient to recapitulate its function. For example, in Kagan *et al* 2006, a construct in which the localization domain of TIRAP was replaced with that of the kinase Fyn could not rescue TLR4 signaling. This construct seems ideal, since Fyn not only localizes to the plasma membrane, but localizes to the same subdomain of the plasma membrane as PI(4,5)P2 [159,249]. However, Fyn's localization domain requires both myristolation and farnesylation, which causes it to integrate with the inner leaflet of

the plasma membrane [250]. These modifications may produce steric hindrance or in some other way prevent TIRAP's association with the TIR domain of the TLR, even if they are in close proximity. In light of this, the results obtained in Figure 2.5B and 2.5C are ideal, since each construct was capable of signaling from either the plasma membrane or endosomes, indicating that negative results cannot be explained by misfolding or inability to interact with TLR TIR domains. Unfortunately, though PS is found at the plasma membrane, it is not found in lipid rafts from which TLR4 initiates signaling, so whether PI(4,5)P2 binding in particular is important is still an open question.

Implications of TIRAP Involvement in pDCs

Our work detailed in Chapter 2 raises other questions as well. We showed that TIRAP was important for initiating signaling from endosomal TLRs in macrophages, as well as to initiate the IFN-producing pathway in pDCs (Figure 2.1). However, there was no defect in the ability of pDCs to produce inflammatory cytokines downstream of endosomal TLRs. As the inflammatory pathway and IFN pathway are initiated from different endosomal compartments (see Chapter 1.5), there are several possible interpretations. The first is that TIRAP is required to recruit MyD88 to the so-called "IRF7 endosome" to trigger production of IFN, but no sorting adaptor is required for the "NfκB endosome to produce inflammatory cytokines. However, it seems increasingly clear that all TIR-domain containing receptors (save TLR3) require a sorting/signaling adaptor pair, including all TLRs studied in macrophages, IL-1/IL-18R and *Drosophila* Toll [160,197]. That later example is especially convincing on this point, since *Drosophila* MyD88 (dMyD88) is the structural homologue of the mammalian signaling adaptor, but

functions in flies as a sorting adaptor. Though dMyD88 has a death domain and could in principal interact with the IRAK homologue pelle, flies have evolved a separate signaling adaptor (Tube) to mediate this interaction. The evolutionary conservation of function, even while the proteins carrying out those functions are diversified, argues strongly that sorting adaptors are critical to these signaling pathways.

A second possibility is that some other sorting adaptor recruits MyD88 to endosomal TLRs in pDCs. Mouse and human genomes contain several TIR domain-containing proteins, many of which are poorly characterized. To date, no one has investigated the role of these proteins in TLR signaling in pDCs. Finally, it is possible that TIRAP is in fact the sorting adaptor for pDCs. The role of TIRAP in endosomal signaling in macrophages was unknown for a decade because of an artifact caused by the use of non-degradable synthetic ligands. Though pDCs in Figure 2.1D were infected with a live virus, it's possible that some artifact of viral dose or cell-culture conditions masked a requirement for TIRAP in those cells. One way to test this hypothesis would be to measure the production of inflammatory cytokines by pDCs in a live viral infection by intracellular cytokine staining. If TIRAP is indeed required, TIRAP knockout pDCs should produce less proinflammatory cytokines in a natural infection.

Further investigation of these distinct pathways in pDCs may be fruitful for understanding the bifurcation of production of inflammatory cytokine and type-1 IFN. This separation is reminiscent of TLR4 signaling in the use of separate pathways for the production of these factors, but differs in at least two respects. First, TLR4 uses an entirely different set of adaptors and signaling enzymes to initiate IFN production, while

endosomal TLRs in pDCs use largely the same components for both pathways (with at least one notable exception discussed below) [76,149,158]. Second, the separation in pDCs cannot be explained by apparent need to restrict IFN production from signals originating at the cell surface (discussed in Chapter 1.5), since all TLR signaling in pDCs is endosomal.

One way to address differences in these pathways in pDCs may be possible by investigating the divergent roles of IRAK1 and IRAK2 [142,143]. As described in Chapter 1.4, while both the inflammatory and IFN-producing pathways in pDCs rely on IRAK4, IRAK1 is used exclusively in the production of IFN, while IRAK2 is required for proinflammatory cytokine production. In fact, these pathways are somewhat mutually exclusive, since IRAK1 knockout pDCs produce more inflammatory cytokines downstream of TLRs than their WT counterparts, while IRAK2 knockouts make more type 1 IFN than WT. Coupled with the knowledge that both of these pathways require MyD88, IRAK4 and TRAF6, this suggests that IFN-producing IRAK1 myddosomes may compete with inflammatory cytokine-producing IRAK2 myddosomes for upstream or downstream components.

It is unclear whether IRAK1 and IRAK2 can be recruited to the same myddosome complex, though this seems unlikely as these pathways are thought to initiate from distinct endosomal compartments[78]. Precisely how IRAK1 and IRAK2 could be differentially recruited to different subcellular locations is unclear. This may be explained by the presence of a sorting adaptor other than TIRAP. However, TIRAP-containing myddosomes in macrophages contain IRAK2 and initiate inflammatory cytokine

production, while TIRAP in pDCs seems to be required solely for the IFN pathway. Intriguingly, the E3 ubiquitin ligase TRAF6, though typically depicted downstream of the IRAKs, is required for both signaling pathways [75,251]. This suggests that the IRAKs may phosphorylate multiple targets, including those downstream of TRAF6. One possibility is that TRAF6 may serve as a "sorting platform" for these independent pathways. TRAF6 contains multiple zinc finger motifs, whose functions are largely uncharacterized [252]. Zinc finger domains function in phospholipid binding, making them ideal localization-determining domains. Further, the closely related TRAF3 is known to exist in several different splice isoforms that are differentially expressed in different cell types, and these isoforms differ principally in their zinc finger domains [253,254]. If TRAF6 also exists in multiple splice forms, this would raise the possibility that TRAF6 may be directed to different subcellular compartments and differentially mediate the recruitment or activation of IRAK1 and IRAK2.

Localization in Other Innate Immune Signaling Pathways

Less is known about the cellular compartmentalization of other PRRs, though some themes are beginning to emerge. Like the bifurcation of IFN and inflammatory cytokine production downstream TLR4 and of TLRs in pDCs, RLR signaling activates separate pathways depending on the localization of its adaptor protein MAVS [62,109]. Unlike TLRs, these pathways do not differ in their ability to produce inflammatory cytokines. Rather, MAVS localized to mitochondria initiates the production of type 1 IFN, while MAVS localized to peroxisomes induces interferon-stimulated genes in a type 1 IFN independent manner. More recent work in our lab (unpublished) suggests that

peroxisomally-localized MAVS induces type 3 IFN. The reason for spatially separating these pathways in most cases is unknown, though one plausible explanation is the ability of cells to differentially regulate these pathways. In the case of TLR4, expression of CD14 is required to move the receptor to endosomes and initiate TRIF-dependent signaling, and cells lacking CD14 are incapable of triggering this pathway in response to LPS [159]. This is true not only in genetically altered mice, but also in cells that naturally lack expression of CD14 such as B-cells. However, no examples of differential spatial regulation of RLR or pDC TLR responses have yet been described.

4.3 Integration of Pattern Recognition Receptor Signals

Relatively little is known about the integration of signals from multiple PRRs, yet most microbes naturally contain several different PAMPs. In addition, the cells of the innate immune system must interpret those signals in the context of cytokines and other signaling molecules produced by neighboring cells. Our work in Chapter 3 demonstrates that at least in some cases, signals downstream of multiple receptors trigger a cell behavior that neither signal induces on its own. In particular, macrophages receiving a cell-intrinsic signal from a MyD88-dependent TLR as well as extrinsic type 1 IFN lose TIRAP, lowering their sensitivity to future microbial encounter. We do not know whether macrophages can initiate this regulatory program in an entirely cell-intrinsic manner. A single ligand (LPS) is capable of triggering loss of TIRAP, but macrophages encountering LPS initiate two signaling pathways, one of which culminates in the production of type 1 IFN (Figure 3.3C). It's possible that this TRIF dependent signal can

provide the second signal in the absence of IFN, though this could be tested by monitoring TIRAP loss in IFNAR knockout macrophages in response to LPS.

While we have not experimentally tested the mechanism of TIRAP loss, our data are consistent with several possibilities (Figure 4.1B). First, the factor responsible may depend on activation signals of both MyD88-dependent TLRs and IFNAR, or on the combined action of transcription factors downstream of both pathways. Second, two or more factors may be responsible for TIRAP loss, with production or activation of at least one dependent on each pathway. Finally, a gene product dependent only on IFN signaling may target TIRAP, but only while it is part of an active signaling complex. Unraveling these possibilities will be challenging, but may reveal new methods of regulation that ultimately influence the immune response. For example, in 2005, multiple groups showed that integration of signals from both arms of TLR4 signaling (TRIF- and MyD88-dependent) led to sustained NFκB activation, rather than an oscillation in activity like that induced by tumor necrosis factor (TNF) [255,256]. However, this did not merely lead to an increase in the levels of gene expression, but rather led to a qualitatively different response.

Understanding the nature and magnitude of the response triggered by pattern recognition receptors is not merely academic, as inflammation lies at the heart of many of the most costly human diseases, from type 2 diabetes to autoimmunity to cancer. Treating disorders characterized by too much inflammation, such as autoimmunity or allergies, is challenging since suppressing the immune system leaves patients vulnerable to infectious assault. At the same time, a remedy for too little inflammation,

as in the case of cancer and some challenging vaccines, is also problematic since excessive inflammation causes tissue damage and may lead to autoimmunity. A more nuanced understanding of activating and inhibitory signals may allow for a clinical fine-tuning of immune responses. Efforts at refining this understanding are already underway. For example, several common genetic variants in human TLR signaling networks cause measurable changes in responses to TLR ligands, which may govern differential responses to infectious challenge or vaccines [257]. Similarly, genome-wide associate screens have identified several PRR-linked genes that increase susceptibility to autoimmune disorders [258-260]. Though the ability to translate these findings to the clinic are uncertain, they highlight the limitations of the traditional genetic approaches that have thus far dominated the investigation of innate immune recognition.

Appendix

Supplemental Figures

Figure S1

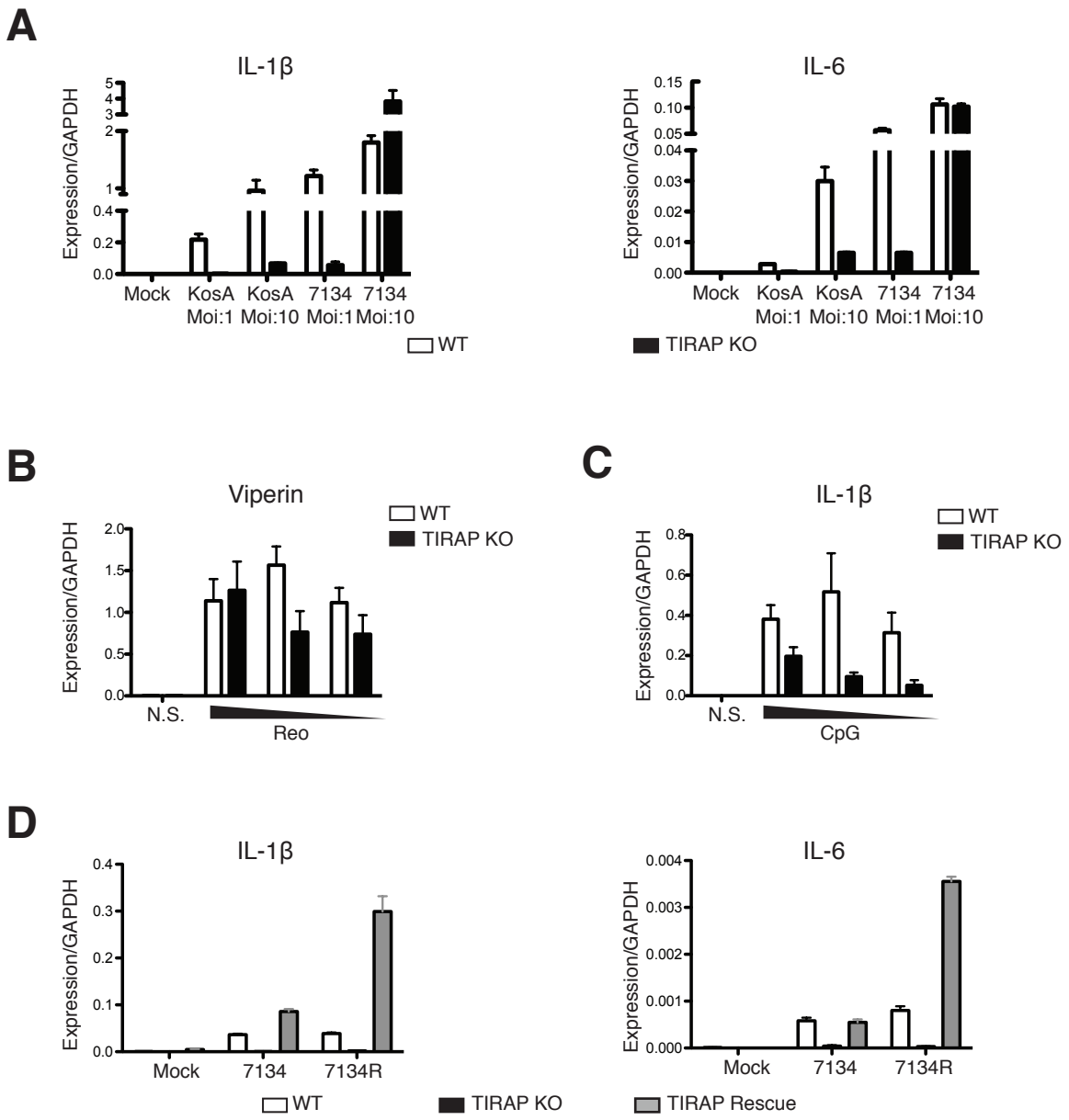

Figure S1.
Further Analysis of TLR Signaling in Primary and iBMDMs, Related to Figures 2.1 and 2.2
(A) WT or TIRAP KO primary BMDM were infected with two strains of HSV at multiple MOIs. Higher MOI with substrain 7134 overcomes the signaling defect in TIRAP KO BMDM.
(B) WT and TIRAP KO primary BMDM were infected with reovirus at multiple MOIs. Responses to reovirus were unimpaired in TIRAP KO BMDM.
(C) WT and TIRAP KO iBMDM were stimulated with increasing doses of CpG DNA (1-100 μM). TIRAP KO cells are defective for cytokine expression except at high ligand concentration, analogous to the concentration-dependence of cell-surface TLRs.
(D) iBMDM were infected with an IPC0 null (7134) or revertant (7134R) strain of HSV and transcriptional responses were assessed by qPCR. Error bars represent SD.

Figure S2

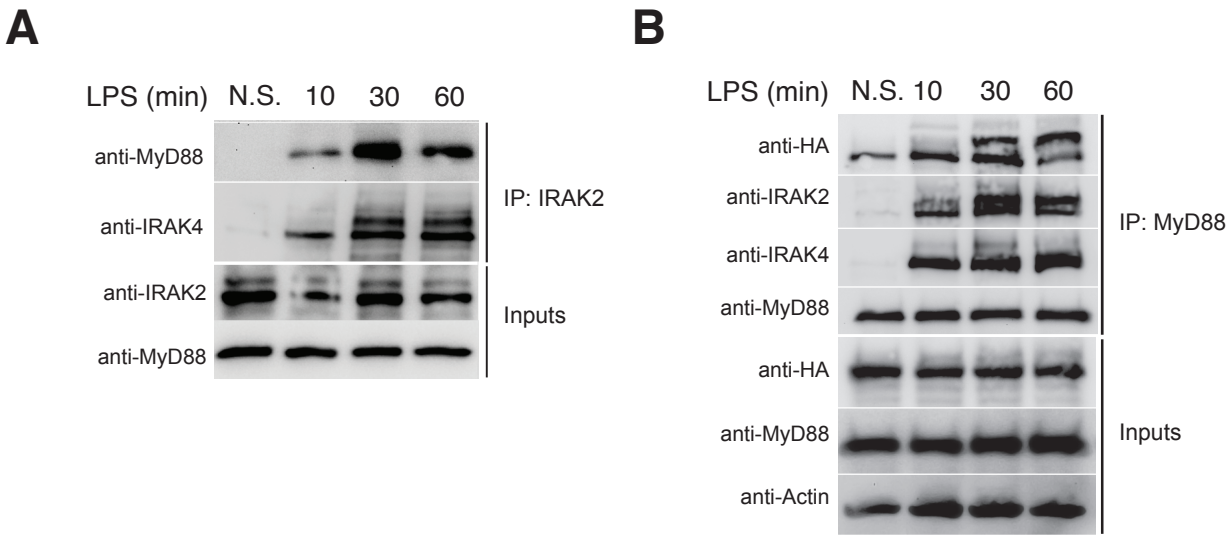

Figure S2.
Biochemical Analysis of TLR-Induced Myddosomes, Related to Figure 2.3
(A) iBMDM were treated with LPS for the indicated times and IRAK2 was immunoprecipitated from lysates and analyzed by western blot.
(B) HA-TIRAP-expressing iBMDM were treated as indicated and MyD88 was precipitated from cleared lysates. Immunoprecipitates were then analyzed by western blot for the indicated proteins.

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